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HANDBOOK OF PRACTICAL BACTERIOLOGY

HANDBOOK OF PRACTICAL BACTERIOLOGY

A Guide to Bacteriological Laboratory Work

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PART I

General Biology of Micro-Organisms and Immunity in Relation to Practical Bacteriology

CHAPTER I

THE GENERAL BIOLOGY OF MICRO-ORGANISMS

THE BIOLOGICAL DIVISIONS REPRESENTED BY THE PATHOGENIC MICROBES

Bacteriology or Microbiology, as applied to medicine, embraces the study of those micro-organisms which are pathogenic to, or commensals of, man. The term "pathogenic" implies the power of producing disease; organisms that occur on the skin or in certain parts of the body—*e.g.* mouth, throat, intestine—without exerting any harmful effect are described as "commensals." Various commensal organisms are, however, potential pathogens, and some recognised pathogenic microbes may, under certain conditions, assume a commensal rôle—*e.g.* in the so-called infection carriers.

In veterinary science, bacteriology is specially concerned with the micro-organisms responsible for disease in domesticated animals. As many infective diseases are common to man and animals, medical and veterinary bacteriology are closely related branches of the general subject. Pathogenic organisms, however, show great diversity in their parasitism to different animal species, certain being associated with disease in the human species only, while others are highly virulent towards particular animals though non-pathogenic to man.

The pathogenic and commensal micro-organisms may be classified broadly in the following biological divisions: (*a*) the bacteria or schizomycetes ("fission fungi"); (*b*) the fungi proper, which include the moulds and the yeasts; (*c*) the protozoa.

The exact biological relationships of some pathogenic micro-organisms, *e.g.* the *Rickettsia* group (which includes the organism of typhus fever), still remain undetermined, and it is difficult to assign such forms to any one of these divisions.

Moreover, certain infective agents, probably of organismal nature, are so minute that they can pass through a filter which is impervious to the recognised bacteria, and are designated "filterable viruses." Some of these are beyond the range of microscopic visibility ("ultra-microscopic"); many, however, have been demonstrated as minute bodies which are smaller than the bacteria. The filterable viruses are still undefined biologically.

The differential characterisation of these biological divisions referred to is as follows:—

BACTERIA.—Morphologically simple, unicellular organisms of microscopic dimensions, multiplying usually with great rapidity and by simple fission; cellular units spherical, cylindrical, comma-shaped, spiral or filamentous; devoid of chlorophyll and exhibiting, as a rule, no "morphological" nucleus; certain species develop a resting-phase in the form of "spores"; some of the filamentous types produce "conidia" (*vide* p. 11); in certain species the cells are motile and possess flagella; some forms are flexuous.

FUNGI: *Mould* forms.—Branching filaments (hyphae) interlacing and forming a meshwork (mycelium); more highly organised than the bacteria, often septate and multicellular, and reproduce usually by means of spores developed in "fruiting organs." *Yeast* forms (saccharomycetes).—Round, oval or elongated units, generally larger than bacteria and multiply by "budding"; in certain species multiple "endospores" formed; in some, hyphae occur.

Protozoa.—Generally regarded as the lowest forms of animal life ; minute unicellular organisms with the protoplasm differentiated into nucleus and cytoplasm ; reproduce by fission and spore-formation, and often exhibit a definite life-cycle with both sexual and asexual phases.

The bacteria and the filterable viruses play the most important part in the causation of human infective disease. Protozoal infections are most prevalent in tropical and subtropical countries.

THE BACTERIA

For practical work some scheme of biological classification is necessary. Various systems have been used, but in medical bacteriology a simple classification, with an expressive nomenclature, serves for all practical purposes. Thus, in the first instance, bacteria can be classified into the following subdivisions :—

HIGHER BACTERIA	**LOWER BACTERIA (or EUBACTERIA)**
Elongated sheathed filaments, often showing true branching; units may be interdependent, some being specialised for reproduction ; more highly organised than the lower bacteria.	Simple unicellular structures, never in the form of sheathed filaments ; each unit equivalent ; many species motile, usually due to their possessing flagella.
Groups of Medical Importance	*Main Morphological Classes*
1. Leptothrices—unbranched filamentous organisms—e.g. *Leptothrix buccalis* (p. 533).	1. Cocci—globose in shape —*e.g.* the streptococci (p. 332).

Groups of Medical Importance—contd.	*Main Morphological Classes—contd.*
2. Streptothrices — filamentous organisms which show true branching, and form a mycelium—e.g. *Actinomyces* (p. 525).	2. Bacilli—straight rod-shaped organisms—e.g. *B. typhosus* (p. 430).
	3. Vibrios and spirilla—definitely curved non-flexuous rods (vibrios) or spirals (spirilla)—e.g. *V. cholerae* (p. 471).
	4. Spirochaetes—flexuous spiral filaments—e.g. *Treponema pallidum* of syphilis (p. 538).

MORPHOLOGICAL STUDY OF THE BACTERIA

Unstained Preparations of Living Organisms.—The morphology of the bacteria can be studied by examining them first in the unstained condition, suspended in fluid. In this way their general shape can be observed and motility determined (*vide* p. 67). Certain very slender organisms, however, such as the spirochaetes, are so feebly refractile that they cannot be seen by the ordinary microscopic methods, and *dark-ground illumination* (*vide* p. 69) is necessary for their demonstration.

For the study of the development of individual organisms and the growth of bacteria in colonies, the "agar-block" method of Ørskov or the microscope-incubator may be used (*vide* p. 188). These methods enable living bacteria to be observed at intervals during their actual growth on a suitable substrate, and present a truer and more natural picture of bacterial morphology than other procedures which may involve such manipulations as to create artificial appearances.

STAINED PREPARATIONS.—The examination of stained organisms is usually an essential routine procedure. For this purpose various dyes are employed, often along with a mordant.

"*Negative*" *staining* is of value for the simple morphological study of bacteria—*i.e.* the organisms are mixed with some substance which, in film preparations, yields a dark or coloured background, while the organisms stand out as clear unstained objects. India ink and nigrosin are examples of substances used for this purpose (*vide* p. 196). Negative staining has also been applied to the demonstration of spirochaetes.

Silver impregnation methods (*vide* p. 221) are utilised for the staining of spirochaetes and are particularly applicable for demonstrating these organisms in tissues.

"Impression preparations" are specially valuable for the microscopic study of organisms in their natural state, *e.g.* in a colony on culture medium. For this purpose whole colonies are transferred to cover-slips and then suitably stained (p. 226).

STAINING REACTIONS.—The staining reactions of the bacteria are of the greatest importance both in their morphological study and for their differentiation and identification. Thus, all the bacteria can be divided into two categories by the so-called Gram's staining reaction (*vide* p. 197)—*i.e.* according to whether they resist decolorisation with aniline oil, alcohol or acetone after staining with a pararosaniline dye—*e.g.* crystal or methyl violet, and subsequent treatment with iodine. Those retaining the dye are designated "Gram-positive"; those decolorised by this method are spoken of as "Gram-negative."

The essential difference between these two categories appears to be that the Gram-positive organisms have the magnesium salt of ribo-nucleic acid as part of their surface structure. By treatment with bile-salt magnesium ribo-nucleate has been extracted from Gram-positive organisms (but not from Gram-negative), leaving a Gram-negative "cytoskeleton," and

Gram-positivity may be restored by recombination between this and magnesium ribo-nucleate (Henry and Stacey, *Nature*, 1943, **151**, 671).

Some bacteria when treated with a strong staining solution resist decolorisation by acid, and are spoken of as "acid-fast"—*e.g.* the tubercle bacillus (*vide* p. 203). This property is generally regarded as due to the lipoid content of these organisms, but may depend on texture as well as chemical composition.

Certain organisms do not stain uniformly and this may be a characteristic feature. Thus, the diphtheria bacillus shows a "beaded" or "barred" appearance when stained with methylene blue. The plague bacillus displays "bipolar staining," the ends being more deeply coloured than the centre.

A staining reaction which is characteristic of certain species—e.g. *B. diphtheriae*—is the appearance of metachromatic granules—*i.e.* certain granules are demonstrable in the bacterial cell, which can be stained with one dye while the rest of the protoplasm stains with a dye of different colour (*vide* p. 206). These structures are sometimes called "volutin" granules. They exhibit a special affinity for nuclear stains and with polychrome methylene blue (*vide* p. 195) are coloured purple. They are apparently rich in nucleo-protein but do not represent nuclear elements, and are probably not vital constituents of the bacterial protoplasm. Their occurrence and number depend on the culture medium on which the organism is growing.

PLEOMORPHISM AND INVOLUTION.—It must be remembered that, especially in artificial culture, bacteria may show considerable variation in shape and size (pleomorphism), and may also exhibit degeneration or "involution" forms which are different morphologically from the normal cell.

MOTILITY.—The motility of bacteria (observed in a fluid medium) is generally due to delicate prolongations of the protoplasm (flagella) which act as locomotory organs. These are not seen in unstained preparations and can be demonstrated only by special staining methods (*vide* p. 212). Brownian movement must not be confused with true motility. In the latter case the organism definitely changes its position in the microscope field (*vide* p. 69). Brownian movement is generally regarded as due to the impact of the molecules of the medium in which the organisms are suspended (" molecular bombardment ") and is an oscillatory movement within a limited radius.

Flagella may be " terminal " (or " polar ")—*i.e.* at one or both ends of the bacterium—and single or multiple. They may be distributed all round the organism, and this arrangement is described as " peritrichous."

When there is a single terminal flagellum the term " monotrichous " can be applied ; " amphitrichous " indicates the presence of a flagellum at each pole ; " lophotrichous " refers to the arrangement of multiple flagella at one or both poles.

Among the spirochaetes motility is generally considered to be a function of the contractility of the cell protoplasm. Flagella, or flagella-like structures, have been demonstrated, however, in certain spirochaetes, e.g. *Treponema pallidum* (*vide* p. 538).

Bacterial Spores.—Some species develop a highly resistant resting-phase or spore, by which the individual survives unfavourable external conditions. The spore is not a reproductive structure. In the vast majority of spore-bearing species only one spore is developed by each vegetative form. This structure appears in the cell protoplasm (" endogenous ") and may increase in size, appearing as a round, oval or elongated body, which is situated in the centre of the bacterium (" central "), at the end (" terminal "), or between the centre and end (" subterminal "). The relative size of the spore varies with different species. Spores can withstand all injurious chemical and physical influences better than the vegetative forms, and probably owe their resistant properties to a dense outer protective membrane and their relatively low water-content. Under favourable external conditions the membrane ruptures and the vegetative form is resumed. This process is described as the "germination" of the spore.

The spore is not stained by the ordinary methods but appears as a clear, unstained portion of the bacterial structure. In some cases, however, the spore stains by Gram's method. Special methods are employed for the differential staining of spores (*vide* p. 208).

It has been found that the antigen (p. 34) of a spore is distinct from that of the vegetative form, which indicates an essential difference in chemical constitution between the two phases.

BACTERIAL CAPSULES.—Certain bacteria may exhibit a relatively thick outer capsule and are described as "capsulated." In certain species complex carbohydrate substances enter into the composition of such capsules, and are of great importance in determining specific characters (*vide* p. 35). Virulence may also depend on capsule formation (*vide* pneumococcus. p. 358). By ordinary methods of staining, the capsule appears as an unstained zone round the organism. Special methods are available for the differential staining of capsules (*vide* p. 210). "Negative" staining is also of particular value for demonstrating capsules (*vide* p. 210).

THE BACTERIAL PROTOPLASM.—No structure corresponding to a nucleus can be defined readily in the bacterial protoplasm. In all probability bacteria possess nuclear material, but either it is scattered in granular form throughout the cytoplasm or the nuclear apparatus exists in some form incapable of exact demonstration by existing methods. It is of interest that the cell protoplasm exhibits an affinity for those basic dyes which are used as nuclear stains.

MULTIPLICATION AMONG THE BACTERIA.—Among the lower bacteria multiplication takes place by simple binary fission. The cell enlarges or elongates, and a constriction develops equatorially or transversely ; this ultimately divides the original individual into two new cells. Division may occur with great rapidity—*e.g.* every half-hour—so that one individual may soon reproduce several millions of new organisms. Among the spirochaetes transverse fission occurs as in other bacteria.

In the higher bacteria transverse division of the filaments into shorter forms is observed. Certain filaments also divide up at their free ends into a number of oval "conidia," which are set free, and each of these, besides representing a resting-phase, may develop a new colony.

Some observers have described more complex processes of reproduction among bacteria, and have regarded the usual forms which multiply by simple fission as one phase only in a life-cycle which they postulate. The evidence at present does not warrant acceptance of such views as applicable generally to the pathogenic bacteria.

COCCI

Cocci are classified morphologically as follows :—

Staphylococcus.—The individuals tend to be grouped in clusters, due to the irregularity of the planes of successive divisions.

Streptococcus.—The cocci are arranged in chains, successive divisions being always in a similar axis.

Tetracoccus.—Plates of four (or multiples of four) cocci, division occurring successively in two planes at right angles.

Sarcina.—Packets of eight (or multiples of eight) cocci, division occurring successively in three planes at right angles.

Diplococcus.—The cells tend to be arranged uniformly in pairs.

The different cocci are relatively uniform in size, 1μ (0·001 millimetre) being the average diameter. Some species are capsulated. Form varies with species, being spherical, oval, lanceolate or kidney-shaped. The reaction to Gram's stain is an important criterion in their identification.

BACILLI

Morphological features of importance in the study and identification of these organisms are :—

Size—some being relatively large—e.g. *B. anthracis* (p. 414), others small—e.g. *B. influenzae* (p. 495).

Shape—rectangular—e.g. *B. anthracis* ; oval (coccobacilli)—e.g. *B. pestis* (p. 478).

Arrangement—in pairs—*e.g.* diplobacillus of Morax (p. 353) ; in chains—e.g. *B. anthracis.*

Occurrence of a capsule—e.g. pneumobacillus.

Motility, flagella and their arrangement—e.g. *B. typhosus* possesses numerous flagella which are peritrichous in arrangement.

Spores, their shape and position—e.g. *B. tetani* is characterised by its spherical terminal spore.

Reaction to Gram's stain (p. 203).

Acid-fastness—e.g. tubercle bacillus.

Staining of cytoplasm—e.g. " beading," bipolar staining, metachromatic granules (*vide supra*).

The bacilli cannot be classified readily into morphological genera, and physiological characters have to be taken into account in their classification. In the past the term " bacillus " has been used as a generic name for all the straight rod-shaped forms, but it must be recognised that such application of the term can hardly be justified in the strict biological sense in view of the great diversity of species which exhibit this morphological form. On the other hand until there is complete agreement on the nomenclature of bacteria the long established use of this generic name in medical and scientific literature would provisionally warrant its continuance.

VIBRIOS AND SPIRILLA

Comma- and S-shaped forms and non-flexuous spirals are characteristic (*vide supra*). Most species are very actively motile (*e.g.* "darting" motility) and the flagella are terminal. They are mostly Gram-negative.

While there is no sharp line of demarcation between the vibrios and spirilla, these organisms have usually been classified into two morphological genera: *Vibrio*—the typically short curved rod-shaped form; and *Spirillum*—the spiral filamentous type.

SPIROCHAETES

Some spirochaetes are relatively large, refractile and easily stained by ordinary methods—e.g. *Borrelia* (or *Spironema*) *refringens* (p. 545); others are delicate, feebly refractile and difficult to stain—e.g. *Treponema pallidum.* Among different species the coils vary considerably in wave-length and amplitude. Movement is undulating or rotatory. Terminal flagella have been demonstrated in some spirochaetes. It has been stated that in certain pathogenic species granules are developed in the protoplasm and extruded from the cell, and these are regarded as a phase in the life history of the organism.

The pathogenic forms can be classified broadly into the following genera:—

(1) *Borrelia*—coils are large and wavy, the wave-length being about 2–3μ, and generally three to seven in number. These organisms are usually more refractile and more readily stained by the ordinary

dye solutions than the other pathogenic spirochaetes. They are exemplified by the relapsing fever spirochaetes (*vide* p. 546) and certain commensal species, such as *Borr. refringens* (*vide* p. 545).

(2) *Treponema*—coils are of shorter wave-length (*e.g.* 1μ), generally regular, sometimes relatively rigid, the organism presenting typically a "corkscrew-like" structure. These spirochaetes are usually feebly refractile and difficult to stain by the ordinary dye solutions. *Tr. pallidum* exemplifies this genus (*vide* p. 538).

(Some systematists classify all the spirochaetes included in these two genera into one composite genus, designated *Treponema.*)

(3) *Leptospira*—sharply twisted filaments with one or both extremities "hooked" or recurved; the coils are fine, closely wound and numerous, and may be difficult to demonstrate except by dark-ground illumination. Spirochaetes of this group, like the *Treponemata*, are feebly refractile and not readily stained by the ordinary methods. They differ from the other genera in being able to survive in water. *L. icterohaemorrhagiae* of infectious jaundice is an example of the genus (*vide* p. 550).

NEWER CLASSIFICATION AND NOMENCLATURE

A system of classification and nomenclature of the bacteria (or Schizomycetes) was introduced some years ago by the Society of American Bacteriologists following strictly the accepted rules of biological classification, and has since been elaborated by American systematists. A brief outline of this system is given here, *but only those orders, families and genera which are of special importance in medical and veterinary bacteriology are dealt with.* The object of this outline is merely to enable the new grouping and nomenclature to be correlated with the older system. Detailed generic characters are not given, but these are indicated in a general way by the various type-species quoted, the characters of which are described in later chapters. It should be noted that this system of classification is based on physiological as well as morphological characters. For full details, the fifth edition of Bergey's *Manual of Determinative Bacteriology* should be consulted.

ORDERS OF BACTERIA (OR SCHIZOMYCETES)

1. **EUBACTERIALES.**—Undifferentiated simple forms, without true branching—spheroidal or rod-shaped, short or long, straight or curved—some motile, due to flagella—non-flexuous—some species produce endospores—some pigmented—some store volutin, glycogen or fat—include many pathogenic species.

2. **ACTINOMYCETALES.**—Mould-like differentiated forms, rod-shaped or elongated and filamentous—may show true branching and formation of mycelium—endospores not produced—conidia may be formed—non-motile—usually Gram-positive—include a number of important pathogenic species.

3. **SPIROCHAETALES.**—Usually slender flexuous spiral forms—endospores absent—include certain important pathogenic species.

4. Chlamydobacteriales
5. Caulobacteriales
6. Thiobacteriales
7. Myxobacteriales

Include no species which are pathogenic to man or higher animals.

EUBACTERIALES

Families:

1. **Nitrobacteriaceae.**
2. **Azotobacteriaceae.**
3. **Acetobacteriaceae.**

Include no pathogens.

4. **Rhizobiaceae.**—Rod-shaped forms—single polar or lateral flagellum or 2–4 peritrichous flagella or non-motile—usually Gram-negative—utilising dextrose and sometimes other sugars without appreciable acid production.
5. **Pseudomonadaceae.**—Straight or spirally curved rod-shaped or elongated forms—usually motile with polar flagella—non-flexuous—Gram-negative.
6. **Micrococcaceae.**—Spheroidal forms—usually non-motile—not arranged in chains—usually Gram-positive—not obligate parasites.
7. **Neisseriaceae.**—Spheroidal Gram-negative forms—generally obligate parasites.

8. **Streptobacteriaceae (or Lactobacteriaceae).**—Spheroidal or rod-shaped units with tendency to chain formation—Gram-positive—decompose sugars with marked acid production.
9. **Parvobacteriaceae.**—Small rod-shaped forms—Gram-negative—aerobic or anaerobic—usually obligate parasites—frequently require body-fluids for growth—not active fermenters of carbohydrates.
10. **Enterobacteriaceae.**—Rod-shaped forms—frequently motile with peritrichous flagella—Gram-negative—active fermenters of various sugars with acid, or acid and gas, production.
11. **Bacteriaceae.**—Miscellaneous non-sporing rod-shaped forms not included in other families.
12. **Bacillaceae.**—Rod-shaped forms—with endospores—usually Gram-positive—aerobic or anaerobic.

Note.—Numbers 6 to 11 are typically heterotrophic organisms (*vide* p. 22).

Genera :

Rhizobiaceae

Alcaligenes.—e.g. *B. faecalis alkaligenes* (p. 457).

Pseudomonadaceae

Pseudomonas.—e.g. *B. pyocyaneus* (p. 348).
Vibrio.—e.g. *V. cholerae* (p. 471).
Spirillum.—Rigid spiral organisms—usually motile with multiple polar flagella—mostly found in water.

Micrococcaceae

Micrococcus.—Cocci occurring in plates or irregular masses—Gram-positive—some produce yellow, orange or red pigment—saprophytes or facultative parasites—type-species is *M. luteus.*
Staphylococcus (p. 327).
Gaffkya.—e.g. *M. tetragenus* (p. 347).
Sarcina.—Cocci arranged in regular packets of eight or multiples of eight (p. 11).

Neisseriaceae

Neisseria.—e.g. meningococcus, gonococcus, etc. (p. 361).
Veillonella.—e.g. *Veill. parvula* (p. 373).

Streptobacteriaceae (or Lactobacteriaceae)

Diplococcus.—e.g. pneumococcus (p. 354).
Streptococcus (p. 332).
Lactobacillus.—e.g. *B. acidophilus* (p. 469).

Parvobacteriaceae

Pasteurella.—e.g. *B. pestis* (p. 478).
Brucella.—e.g. *Br. melitensis* (p. 488).
Malleomyces.—e.g. *B. mallei* (p. 410).
Haemophilus.—e.g. *B. influenzae* (p. 495).
Noguchia.—e.g. *Bact. granulosis* (p. 629).
Dialister.—e.g. *Bact. pneumosintes* (p. 501).

Enterobacteriaceae

Escherichia.—e.g. *B. coli communis* (p. 424).
Aerobacter.—e.g. *B. lactis aerogenes* (p. 427).
Klebsiella.—e.g. pneumobacillus (p. 428).
Proteus.—e.g. *B. proteus* (p. 348).
Eberthella.—e.g. *B. typhosus* (p. 430).
Salmonella.—e.g. *B. enteritidis* (p. 445).
Shigella.—e.g. *B. dysenteriae* (p. 452).
Serratia.—e.g. *S. marcescens* (*B. prodigiosus*, p. 597).

Bacteriaceae

Listerella.—e.g. *Bact. monocytogenes* (p. 393).
Actinobacillus (p. 531).
Bacteroides.—e.g. *B. fragilis* (p. 470).
Fusobacterium.—e.g. *B. fusiformis* (p. 536).
Bacterium.—Generic term retained for non-sporing, rod-shaped forms whose taxonomic position has not yet been definitely established.

Bacillaceae

Bacillus.—e.g. *B. anthracis* (p. 414).
Clostridium.—e.g. *B. tetani* (p. 504).

ACTINOMYCETALES

Families :

1. **Actinomycetaceae.**—Filaments, often branched and forming mycelium—conidia sometimes produced.
2. **Mycobacteriaceae.**—Rod-shaped, rarely filamentous—only occasional branching—no conidia.

Genera :

Actinomycetaceae

Leptothrichia.—e.g. *Leptothrix buccalis* (p. 533).
Actinomyces (p. 525).
Erysipelothrix.—e.g. *B. rhusiopathiae* (p. 533).

Mycobacteriaceae

Mycobacterium.—e.g. *B. tuberculosis* (p. 394).
Corynebacterium.—e.g. *B. diphtheriae* (p. 374).

SPIROCHAETALES

Genera :

Borrelia.—e.g. *Borr. recurrentis* (p. 546).
Treponema.—e.g. *Tr. pallidum* (p. 538).
Leptospira.—e.g. *L. icterohaemorrhagiae* (p. 550).

At present there is no general acceptance of any standard system of classification or nomenclature. However, much of the classification and many of the names given above are coming into general use, though older designations are still being applied in medical and bacteriological literature. There is also diversity in the use of particular names. Thus, the generic name *Bacterium* is often applied in bacteriological literature in this country to the organisms which have been described as the " coli-typhoid " group, i.e. *Escherichia*, *Aerobacter*, *Klebsiella*, *Eberthella*, *Salmonella* and *Shigella*, though in the American classification the generic name *Bacterium* is now given only to certain Bacteriaceae whose taxonomic position has not yet been defined. The latter seems the more appropriate usage. It is, of course, customary to speak of all the schizomycetes as " bacteria." In this book the older conventional designations are retained but new names are also given.

PHYSIOLOGY OF THE BACTERIA

The physiology and biochemistry of bacteria can be studied by observations made with " cultures " growing in the laboratory in a nutrient medium.

Bacteria are subject, as regards their growth and vitality, to various external influences—*e.g.* atmosphere, temperature and moisture of their environment, light, nutrient and other chemical substances, etc..

ATMOSPHERE.—Some species grow most abundantly in the presence of *free* oxygen—*i.e.* when exposed to air—and are described as " aerobes "—e.g. *B. tuberculosis*. Others fail to grow in the presence of *free* oxygen, " obligatory anaerobes "—e.g. *B. tetani*. Evidence has been obtained that in the presence of free oxygen these organisms form hydrogen peroxide which is inhibitory to their growth, and unlike certain other peroxide-forming bacteria do not produce catalase (*vide* p. 172). The majority of pathogens are, however, indifferent as regards atmospheric conditions and will flourish in either the presence or absence of oxygen ("facultative anaerobes"—e.g. *B. typhosus*). Certain pathogenic bacteria grow best in the presence of a trace only of *free* oxygen and are designated "microaerophile"—*e.g.* one type of the *Streptothrix actinomyces*. An atmosphere with a certain concentration of carbon dioxide (5–10 per cent.) is essential for the growth of some bacteria, *e.g.* the bovine type of *B. abortus* when first isolated from the body.

Recent studies have shown that bacterial respiration depends on complex oxidative mechanisms, the consideration of which is beyond the scope of this book. For further information on the subject, reference should be made to one of the larger works.

TEMPERATURE.—(a) *Influence on Growth.*—For each species there is a definite temperature range within which growth takes place. The limits are the "maximum" and "minimum" temperatures, and an intermediate "optimum" temperature can usually be recognised at which the best growth occurs. The optimum temperature of a bacterium is approximately that of its natural habitat—*i.e.* about 37° C. in the case of the micro-organisms which are pathogenic to man. Some organisms have a wide temperature range (*e.g.* 5°–44° C.), others are more restricted (*e.g.* 25°–40° C.). Below the minimum, viability is not necessarily interfered with, but above the maximum death more or less quickly ensues, except in spore-bearing species.

Some species of saprophytic bacteria grow best at remarkably high temperatures (*e.g.* 60°–70° C.). These are designated *thermophile*.

(b) *Influence on Viability.*—Heat is an important agent in the artificial destruction of micro-organisms, the effect depending on coagulation and denaturation of cell proteins. In general, among the bacteria which are parasites of mammalian animals, non-sporing forms, in the moist state, cannot withstand temperatures above 45° C. for any length of time.

The "*thermal death-point*" is defined as the lowest temperature (above the maximum for growth) which kills a particular organism in a given time—*e.g.* ten minutes. Thus, the thermal death-point of the pneumococcus is approximately 52° C..

Bacteria are more susceptible to moist than dry heat. Spores are much more resistant to heat than vegetative bacteria, but their degree of resistance varies in different species. Further data on this subject are given in later chapters.

Some species die if kept at 0° C., but others may survive much lower temperatures.

MOISTURE.—Four-fifths by weight of the bacterial cell consists of water, and, as in the case of other organisms, moisture is necessary for growth. Drying in air is generally injurious; spores, however, can resist this influence for long periods—*e.g.* spores of *B. anthracis*, for several years. Certain non-sporing bacteria also may exhibit considerable resistance to drying—*e.g.* the tubercle bacillus (*q.v.*). If cultures of non-sporing bacteria are rapidly dried and kept *in vacuo* in the dark they may survive for long periods, even for several years.

LIGHT.—The optimum condition for growth and viability is darkness. Ultra-violet rays are markedly bactericidal—*e.g.* direct sunlight or radiation from an arc or mercury-vapour lamp.

ANTISEPTICS, AND CHEMOTHERAPEUTIC SUBSTANCES.—A great variety of inorganic and organic chemicals are bacteriostatic (inhibit bacterial growth) or bactericidal (kill bacteria), depending on the concentration brought into contact with the particular organism.

Substances possessing marked bacteriostatic or bactericidal properties are usually designated *antiseptics*, and their practical applications are now well known. Various chemical substances exhibit antiseptic action and have been used for that purpose: *acids—e.g.* boric acid; *alkalis—e.g.* sodium hydroxide; *metallic salts—e.g.* perchloride and biniodide of mercury; *organic metallic compounds—e.g.* merthiolate; *halogens—e.g.* chlorine (as derived from bleaching powder, etc.), iodine; *alcohols* and *ethers—e.g.* ethyl alcohol; *aldehydes—e.g.* formaldehyde; *cyclic hydrocarbons—e.g.* benzol; *cyclic alcohols—e.g.* phenol, cresol; *volatile oils and their products—e.g.* thymol; *oxidising agents—e.g.* hydrogen peroxide; *reducing agents—e.g.* sulphurous acid; various *dyes—e.g.* brilliant green, proflavine, etc..

These various antiseptic chemicals may exhibit considerable differences in their intensity and rapidity of action. Moreover, bacterial species may differ greatly in susceptibility to particular antiseptics; this will be illustrated later in connection with selective methods of cultivation.

It should be noted that even *neutral salts*, such as sodium chloride, may exert bacteriostatic or bactericidal properties in certain concentrations.

Certain chemical substances are successfully used for therapeutic purposes in bacterial and other infections in virtue of their capacity of directly or indirectly controlling the development of the particular micro-organism in the blood or tissues. These substances, however, in the concentrations in which they can be applied in the body may not directly kill the parasitic organism though they may interfere with its metabolism, inhibit growth or reduce virulence, thus allowing the natural defences of the body to play their full part in combating the infection. This is exemplified by the *sulphonamide compounds* (sulphanilamide, sulphapyridine, sulphathiazole, etc.). Various "*antibiotic*" substances (p. 23) derived from plants, fungi and saprophytic bacteria are potent bactericidal or bacteriostatic agents, *e.g.* penicillin. The properties and application of these substances are referred to in later chapters and in the **Appendix.**

ELECTRICAL INFLUENCES.—Bacteria may be killed by the passage of an electrical current through the medium inwhich they are contained. This is not due to any direct electrical effect. In the case of an alternating current, the resultant heating effect may be bactericidal *per se*, and, if a direct current is used, electrolytic changes with the liberation of acid, nascent oxygen or chlorine may lead to bactericidal effects of a chemical nature.

FOOD SUPPLY.—The growth of bacteria is, of course, dependent on an adequate supply of suitable food material. This varies with the natural adaptations of different species. Thus, the general rule has been followed, in the artificial culture of the pathogens, that the medium should approximate, as far as possible, to the composition of the tissues and body fluids (*vide* p. 91). Certain bacteria can be cultivated in a variety of food media ; others are highly restricted in their special food requirements. All bacteria require sources of nitrogen, carbon and the other elements which enter into the constitution of living matter. Some saprophytic forms utilise inorganic nitrogen and the carbon dioxide of the atmosphere like plants, and are designated *autotrophic*. The pathogenic bacteria generally require organic nitrogenous and carbonaceous food material, *e.g.* amino-acids and carbohydrates. To such organisms the term *heterotrophic* is applied. Some of these, nevertheless, are able to synthesise amino-acids, *e.g.* tryptophane, from ammonium salts if suitable organic carbonaceous material is also available. This subject is dealt with in detail from the practical standpoint in Chapter IV. The *H-ion* *concentration of the medium* is also an essential factor in influencing growth (*vide* p. 110). The majority of bacteria grow best at a slightly alkaline reaction.

MUTUAL INFLUENCES.—Different bacteria may flourish well together, the presence of one species favouring the growth of another—*symbiosis*—*e.g.* staphylococci favour the growth of *B. influenzae.* The reverse effect may also be observed—*antibiosis*—where one species is antagonistic to another. This phenomenon is dealt with in the **Appendix.**

BACTERIAL ENZYMES.—In the metabolism of bacteria and in the biochemistry of bacterial action enzymes play an essential part. One of the great functions of bacteria in nature is to produce chemical decomposition of complex organic substances—*e.g.* proteins and carbohydrates—by means of their enzymes. Among the pathogens, fermentative properties are important features in the identification of certain species. Thus, various bacteria decompose particular carbohydrates (*e.g.* sugars, hexahydric alcohols, polysaccharides) with the formation of acids (*e.g.* formic, acetic and lactic acids) and, in many cases, gases (*e.g.* carbon dioxide and hydrogen). Certain species are also capable of breaking down salts of the organic acids.

Some bacteria possess marked proteolytic properties and digest complex protein substances, such as gelatin, coagulated serum, etc., liberating the various products of protein disintegration—*e.g.* amino-acids, ammonia compounds, indole, skatole, hydrogen sulphide, etc.. (See also p. 94.)

CHROMOGENESIS.—Some bacteria, including certain pathogens, produce characteristic pigments, *e.g.* yellow, green, etc., which in some cases diffuse readily from the bacterial cells into the surrounding medium.

GROWTH PHASES.—When bacteria are introduced into a sterile culture medium, their growth proceeds as follows: there is first a period of "lag" (*e.g.* two hours) during which there is no increase in number or only slow multiplication; this is followed by a phase of rapid multiplication (*e.g.* during seven to eight hours at optimum temperature); the increase in number is in geometric progression and the logarithmic curve of growth is a straight line; this is called the "logarithmic" phase; then follows a "stationary" phase, multiplication gradually ceasing; in this phase (lasting from a few hours to several days) the number of new individuals tends to be equalled by those dying; finally there is a phase of decline or diminution of viable individuals, the organisms progressively dying until (after several days to months) no living bacteria remain. It should be noted that the initial "lag" phase may be absent when fresh medium is inoculated with organisms which are already in the phase of multiplication. The "lag" period is probably due to the fact that the organisms after being injured by the products of their previous growth require to undergo "rejuvenation" before they begin to divide and multiply.

BACTERIAL TOXINS.—These are defined as the products of bacteria which are injurious to the tissues and in virtue of which disease processes result from bacterial infection. They have generally been regarded as of protein nature but it has proved exceedingly difficult to isolate them in a state of purity and. in general, their true chemical constitution remains undetermined. In some cases complex carbohydrates and phosphatides may enter into the composition of bacterial toxins.

They are classified broadly as:—

(1) Extracellular toxins, or *exotoxins*, which diffuse readily from the bacteria into the surrounding medium.

(2) Intracellular toxins, or *endotoxins*, which are retained within the cells until the bacteria die and disintegrate.

The majority of the pathogens produce endotoxin only, but some develop powerful exotoxins—e.g. *B. diphtheriae*, *B. tetani*, *B. botulinus*, certain types of staphylococci and streptococci.

Exotoxins are generally unstable substances, their toxic effect being annulled readily by chemicals. free oxygen, and heat—*e.g.* in the case of the diphtheria toxin, at 65° C.. They tend to be highly specialised in their action on particular tissues. Preparations of these toxins can be obtained by growing the bacteria in fluid culture which is then filtered through an earthenware or other bacterial filter—the filtrate contains the toxin (*vide* pp. 88, 378). After introduction into the body there is usually a short incubation period (*e.g.* some hours) before symptoms of poisoning are manifest. By immunising animals with such preparations a specific neutralising antibody (antitoxin) is developed which can be demonstrated in the blood serum of the immune animals (*vide* p. 36).

Most pathogenic organisms do not produce such diffusible toxins, and their culture-filtrates are non-toxic ; on the other hand, their dead bodies are toxic, and it is assumed that their poisons are bound up in the bacterial cell—hence the designation endotoxin.

These toxins are less specialised in their action and produce a more general poisoning effect. They are more stable than exotoxins and can withstand a temperature of 100° C.. They can be demonstrated experimentally by injecting dead cultures or bacterial extracts into animals. There is no definite incubation period following their introduction as in the case of the exotoxins. Immunisation with endotoxins does not lead usually to the formation of antitoxin.

Certain toxins are designated according to their particular effects, irrespective of the organism producing them—e.g. *haemolysin* (producing lysis of red blood cells), *leucocidin* (destructive to leucocytes), *necrotoxin* (producing necrosis of tissue).

Some pathogenic bacteria neither produce exotoxin in culture nor are their dead bodies definitely toxic—e.g. *B. anthracis.* It is probable that such organisms can produce their toxins only when growing in the tissues.

Many pathogenic bacteria, especially when growing in the tissues, form substances which increase their aggressiveness (*vide infra*), and this type of product has been designated *aggressin*. It is supposed that it acts mainly by interfering with opsonisation and phagocytosis (*vide* p. 39), and that by its agency bacteria are able to break down the first defences of the tissues. Various bacterial products, however, may possess aggressin-like properties, and the existence of a specialised substance of this type is doubtful.

VIRULENCE OF MICRO-ORGANISMS.—Virulence is an important property of micro-organisms in relation to their pathogenic action, and is defined as the capacity to invade the tissues, multiply and produce toxic effects. Virulence is estimated by the *minimum lethal dose*—*i.e.* the smallest dose of the organism (*e.g.* in the form of a culture) which will kill a particular species of animal. As a result of the varying susceptibility of individual animals to bacteria and their toxins, it is often impossible to state the exact minimum dose, and it is now customary to refer to the *average lethal dose* for a number of individual animals. In other parts of this book the symbol M.L.D. (minimum lethal dose) may be taken to denote the average lethal dose. It must be noted that virulence depends on two factors which may be quite independent of one another: the invasive power or aggressiveness, and the toxigenic property of the organism. Thus, the tetanus bacillus is highly toxigenic but only weakly aggressive; the anthrax bacillus is markedly aggressive but its toxicity is relatively less pronounced.

The virulence of an organism can be either "exalted" or "attenuated" artificially.

Exaltation of virulence may be produced by passing the strain through a series of individuals of the same species, inoculating the animals one from another in succession—*i.e.* "passage." In this way the virulence is increased for that particular species.

Attenuation usually results when organisms are cultivated artificially for some time; thus, stock laboratory cultures are usually of low virulence, as compared with recently isolated strains. Other methods of lowering virulence are referred to on p. 32.

Variation and Dissociation.—Bacterial cultures may, under certain conditions, " dissociate " and develop variants which differ in certain respects from the original strain—*e.g.* morphological features, absence of motility, loss of capsules, the characters of colonies on culture medium, fermentative and other biochemical properties, virulence, antigenic characters as indicated by serological reactions, etc. (*vide* p. 28). The variant strain may retain the new feature or property as a stable character. Such dissociation is met with in various groups but has been specially investigated among the Gram-negative bacilli occurring as commensals or pathogens in the intestine (*vide* Chapter XV). For example, a strain of coliform bacillus may at first fail to ferment lactose (*vide* p. 427) but develop a lactose-fermenting variant. In colonies on solid media such variants may appear as small papillae. Variation in the physical characters of colonies may also be noted in pure cultures of certain organisms—*e.g.* the " smooth " (S) and " rough " (R) colonies as seen in the typhoid-paratyphoid and other groups. These differences in colony-characters are associated with difference in virulence, antigenic constitution and immunising properties. Thus, the rough (R) variant of the pneumococcus is avirulent and this variation is associated also with absence of capsule-formation and loss of a specific chemical constituent present in the capsule (*vide* p. 358). Motile bacteria may develop non-motile variants. The relationship of these variations to antigenic characters is dealt with later in connection with agglutinating antibodies (*vide* p. 41). For further information on this subject one of the larger works should be consulted.

SYSTEM OF IDENTIFICATION OF THE BACTERIA

(1) The Morphology, together with the Staining Reactions, of Individual Organisms generally serves as a preliminary criterion, particularly for placing an unknown species in its appropriate biological group. In medical bacteriology the microscopic characters of certain organisms in pathological speci-

mens may be sufficient for diagnostic identification—*e.g.* tubercle bacilli in sputum. Morphology among the bacteria usually fails, however, to differentiate allied organisms—*e.g.* meningococcus, gonococcus and *Diplococcus catarrhalis.*

(2) CULTURAL CHARACTERS, OR THE MORPHOLOGY OF GROWTHS ON CULTURE MEDIUM—*e.g.* the appearances of " colonies " to the naked eye or with certain magnifications. This criterion is important in identification, but may also be insufficient to differentiate species—*e.g.* different species of the typhoid-paratyphoid and dysentery groups produce indistinguishable colonies.

(3) PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERS—*e.g.* the fermentation of various carbohydrates. Species which cannot be distinguished by morphology and cultural characters may exhibit distinct differences in their biochemical reactions—*e.g.* typhoid-paratyphoid group. Different species or types may, however, resemble one another closely in fermentative properties—e.g. *B. paratyphosus* B and *B. aertrycke.*

(4) SEROLOGICAL REACTIONS.—In bacteriology, species and types can often be identified by specific "antibody reactions." These depend on the fact that the serum of an animal immunised against a micro-organism contains specific antibodies (for the homologous species or type) which react in a characteristic manner with the particular organism (*vide* p. 36). Such antisera, for example, may agglutinate or clump the homologous organism in test-tube experiment, and this effect can be observed easily with the naked eye.

It should be noted here that the serum of a person or animal suffering from a bacterial infection may also exhibit specific antibody reactions, and in this way the agglutination reaction can be used for diagnostic purposes—*e.g.* the Widal reaction in enteric fever.

(5) ANIMAL EXPERIMENT.—In the case of patho-

genic organisms—e.g. *B. tuberculosis*—which are virulent to, and produce characteristic lesions in laboratory animals, the inoculation test provides a reliable method of identification.

FUNGI ; PROTOZOA

The general morphology and physiology of these organisms will not be dealt with here. The biological aspects of the groups that are of special importance in practical bacteriology as applied to medicine will be referred to later in the consideration of particular organisms (Chapters XXII and XXIII).

FILTERABLE VIRUSES

Little is yet known regarding the biology of these viruses. It has been generally assumed that they are living organisms, owing to the fact that they can be propagated through a series of animals. Many of them, though demonstrable as viruses by the experimental method, have not been observed microscopically, and the designation "ultramicroscopic" has often been used. In certain cases, however, exceedingly minute bodies (" elementary bodies ") within the limits of microscopic visibility have been defined as the actual virus. So far none of these filterable viruses has been cultivated like the bacteria, *i.e.* on a substrate of inanimate material, though they may flourish *in vitro* in association with living tissue cells, *e.g.* a tissue culture.

It should be noted that certain very minute bacteria which are easily recognised by ordinary microscopic methods and are quite readily cultivable may pass the coarser types of filter, e.g. *Bacterium pneumosintes* (*vide* p. 501), and the organism of bovine pleuropneumonia (*vide* p. 535).

The filterable viruses are easily inactivated by various physical and chemical agencies, *e.g.* heat and antiseptics, but on the whole are more resistant than the non-sporing bacteria. They often possess a high degree of resistance to glycerol.

In general their demonstration and identification depend on the experimental production of a characteristic pathological condition in animals (or man) by means of filtrates after subjecting material from the particular disease (*e.g.* nasal washings, serum) to filtration through a filter capable of arresting the ordinary bacteria (*vide* p. 88).

The subject of the filterable viruses is dealt with further in Chapter XXIV.

CHAPTER II

IMMUNITY IN RELATION TO PRACTICAL BACTERIOLOGY

THE subject of immunity is intimately related to practical bacteriology, and immunological principles underlie certain bacteriological methods.

The term "immunity," in its usual application, signifies the power of the animal body to resist (*a*) infection by parasitic micro-organisms,[1] or (*b*) the injurious effects of their products or toxins.

Immunity may be *acquired*, as in the natural recovery from infection, and is due to the development, during the illness, of *specific* resisting powers against the causal organism or its toxin. Once developed, acquired immunity may persist for long periods, even practically throughout life, as exemplified by the immunity following smallpox. On the other hand it may be transient—*e.g.* after pneumonia.

An acquired immunity may also be developed artificially by inoculating an animal with a micro-organism or toxin in such modified form that it is incapable of reproducing the typical disease though still able to bring about an immunity reaction. Such immunity is described as an *active artificial immunity*.

A modified form of micro-organism used for immunisation has generally been designated a *vaccine*, and the inoculation of it is described as *vaccination*, these terms being derived from Jenner's method of inoculating vaccinia virus for preventive immunisation against smallpox (*vide infra*).

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[1] The terms "micro-organism" and "organism" used in this chapter on immunity apply also to the viruses (*vide* p. 29).

The blood serum of an actively immunised animal injected into the body of a non-immune animal renders the latter temporarily immune, and this state is termed *passive immunity*.

The most frequent *methods of producing active artificial immunity* are :—

(1) Introduction (into the body) of living organisms in a state of attenuated virulence. Various methods have been used to reduce virulence for purposes of immunisation—*e.g.* (*a*) subjecting them to drying, as in Pasteur's method of attenuating the rabies virus (present in the spinal cord of experimentally infected rabbits) ; (*b*) cultivating at a temperature above the optimum, as in the case of Pasteur's anthrax vaccine ; (*c*) passage through animals of a different species ; thus vaccinia (cow-pox) virus (as used for smallpox vaccination) may be regarded as a form of the smallpox virus attenuated for the human species by its propagation in bovines ; (*d*) continued cultivation in the presence of an antagonistic substance ; thus, the " B.C.G." vaccine advocated for immunisation against tuberculosis is an avirulent strain of the tubercle bacillus attenuated by prolonged cultivation on a medium containing bile.

(2) Introduction of organisms—usually in the form of cultures—killed by heat or antiseptics—*e.g.* the usual form of *bacterial vaccine*. Repeated and increasing doses are administered. This system is applied in the prophylaxis of certain infections (*e.g.* anti-typhoid vaccination), in therapeutic immunisation (*e.g.* treatment of chronic infections with vaccines), and also in the preparation of antibacterial sera in animals. Viruses killed or inactivated by antiseptics—*e.g.* phenol—have also been used for immunisation.

Immunity may be produced in some cases by means of bacterial extracts which illustrates the fact that the immunising stimulus depends on a chemical constituent of the bacterial cell (*i.e.* the "antigen"—*vide infra*), and a further development in artificial immunisation has been the use of purified preparations of the specific antigen extracted from bacterial cultures.

In *immunising animals* the process may be started with dead organisms, followed by doses of virulent living organisms. In some cases, immunisation is carried out by giving non-lethal doses of unaltered living organisms without previous injection of dead or attenuated organisms, but this method may be difficult to control. A further method is to introduce living organisms and at the same time a specific antiserum which affords the necessary protection against them.

(3) Introduction of exotoxins in repeated and increasing doses, starting with small and harmless doses, and graduating the series, so that, as the immunity progressively develops, each dose is devoid of harmful effect. This is exemplified in the preparation of diphtheria and tetanus antitoxins by immunising animals—*e.g.* horses—with the respective toxins. To ensure that no injurious effects may result from immunisation, toxin which has been rendered non-toxic (*toxoid* or *anatoxin*) by certain chemicals—*e.g.* formalin, etc.—or by heat, or has been partially neutralised by antitoxin, is frequently used. Thus, immunisation with diphtheria toxoid has been extensively applied in the prophylaxis of diphtheria in the human subject (*vide* p. 384).

In some cases the treatment of a culture of a toxigenic sporing bacterium, e.g. *Vibrion septique*, with formalin in certain concentrations may yield a most effective vaccine for immunising animals against the particular infection; the organisms are killed and the toxin is converted to toxoid. This type of vaccine has sometimes been designated *anaculture.*

The injection of an exudate rendered free from bacteria by filtration (the so-called "aggressin," *vide* p. 26) may, in certain cases, be used to immunise animals against the organism responsible for the infection from which the exudate is obtained.

Such "aggressins" have been used in the control of particular animal diseases, *e.g.* blackleg (*vide* p. 524).

The serum of an actively immunised animal is designated an *immune serum* or *antiserum*, and owes its effect to specific *immune bodies* or *antibodies* which act adversely on the homologous organism or neutralise its toxins.

The special constituent or product of the organism which incites antibody production is termed an *antigen* and is generally of protein nature though other substances such as carbohydrates may enter into the composition of bacterial antigens.

A serum containing antibodies antagonistic to the particular bacterium is spoken of as an *antibacterial serum*; one containing antibodies which neutralise toxin, as an *antitoxic serum.*

It has to be noted that, apart from bacteria and their toxins, other foreign cells, and in fact *all soluble complete protein substances foreign to the animal body, may act as antigens* and incite specific antibody production—*e.g.* red blood corpuscles, blood serum, animal venoms, etc..

Thus, the red corpuscles of one species injected into an animal of different species incite the development of an antibody which, under certain conditions, can effect lysis of the red cells of the former. This antibody is described as a *haemolysin* and the serum containing it as a *haemolytic antiserum.*

As a general rule, *an antigen, to produce immunity, must be injected parenterally—i.e.* by some route other than the alimentary tract (subcutaneously, intravenously, etc.).

Usually an animal can be immunised only against an antigen which is foreign to its own tissues. Thus, a rabbit can be immunised against ox or sheep red cells, but not against the cells of its own species.

Certain non-protein substances—*e.g.* lipoids, carbohydrates, etc.—though unable to act as antigens *in vivo*,[1] may when combined with protein be capable of inciting the formation of antibodies which *in vitro*[1] react specifically with the non-protein substance—*e.g.* in the precipitation or complement-fixation reaction (*vide* p. 37). To these substances the term *hapten* is applied. The specific characters of the antigen of a bacterium may, in fact, depend on a non-protein constituent—*e.g.* a polysaccharide as in the case of the different types of the pneumococcus (*vide* p. 358), and groups of haemolytic streptococci (*vide* p. 338).

The action of haptens may be represented schematically as follows:—

$$\begin{tikzcd}
\text{[Carbohydrate-protein]} \atop \text{(antigen)} \arrow[r, "\textit{in vivo}"] \arrow[d, equal] & \text{Animal (immunised)} \arrow[d] \\
\text{Carbohydrate} \atop \text{(hapten)} \arrow[r, leftrightarrow, "\textit{in vitro}", "\text{specific precipitation reaction}"'] & \text{Specific antibody (in serum)}
\end{tikzcd}$$

Specificity is one of the pronounced characters of acquired immunity and of antibodies, and is usually for the biological species, as in the case of *B. typhosus* (*vide* p. 432), though in certain organisms it may be more restricted—*e.g.* to "types" within the supposed species, such as the serological types of the pneumococcus (*vide* p. 356).

The specificity of antibodies depends on the specificity of the corresponding antigens. An organism may contain more than one antigenic constituent, and for each of these, on immunisation, a separate antibody is produced. When related bacterial species have certain antigenic constituents in common, as may be the case, an antiserum for one of these species exhibits to a greater or less degree *group action* towards the others.

It must be remembered that, in some instances, the occurrence of antibodies may have no aetiological significance. Thus, in typhus fever a serum-antibody is demonstrable which is specific for a particular type of *B. proteus*—though this organism has no aetiological relationship to the disease (*vide* p. 559).

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[1] "*In vivo*" denotes "in the living body"; "*in vitro*" (literally, "in glass") means "in test-tube experiment."

Specific antibodies may also occur naturally : thus, the serum of the guinea-pig contains a haemolytic antibody for ox red corpuscles ; specific bactericidal and agglutinating antibodies (*vide infra*) for various bacteria may be demonstrated in the serum of normal animals.

Antitoxic sera are produced by immunising an animal with exotoxins. For example, " diphtheria antitoxin " is the serum of a horse which has been immunised with graded doses of diphtheria toxin. When appropriate amounts of toxin and antitoxin are mixed together the mixture is non-toxic. This process of neutralisation is complex and need not be dealt with here. It occurs both *in vivo* and *in vitro*.

The antitoxic principle is associated with the globulin of the serum ; by a process of precipitating the globulin with half-saturated ammonium sulphate solution, separating the precipitate, dialysing out the ammonium sulphate, and then redissolving the precipitated globulin in a quantity of physiological salt solution much less than the original volume of the serum, an antitoxic serum can be " concentrated " and " refined." Other methods of concentrating and refining antisera have also been used, *e.g.* precipitation by diluting serum with a large bulk of distilled water or weakly acid buffer solutions (Felton). Recently " refined " antitoxins have been obtained by treating the serum with proteolytic enzymes (*vide* p. 382).

Endotoxins unlike exotoxins do not as a rule incite the production of antitoxins.

Antibacterial sera are generally produced by immunising with the actual bacteria, or bacterial extracts. These sera may exhibit the following properties :—

Bacteriolytic or *bactericidal*—*i.e.* directly lysing or destroying the bacteria.

Opsonic or *bacteriotropic* — *i.e.* rendering the bacteria susceptible to phagocytosis.

Agglutinating—immobilising and clumping the bacteria.

Precipitating or producing a precipitate along with the soluble products of the bacteria, *e.g.* a bacterial extract.

Complement-fixing—*i.e.* along with the bacterial antigen "fixing" or "absorbing" complement, a normal constituent of serum (*vide infra*).

These effects may be demonstrated *in vitro*. They are all relatively specific for the particular organism and are quantitatively marked—*e.g.* they may be produced by exceedingly minute amounts of the antigen or antiserum.

The serum of a person or animal infected with a pathogenic bacterium may exhibit also similar properties and constitutes an antibacterial serum. Thus, the serum of a person suffering from typhoid fever exhibits these properties when tested *in vitro* with the typhoid bacillus.

It has to be noted that normal serum may possess bactericidal, opsonic and agglutinating effects. These are relatively weak as compared with the corresponding effects produced by an immune serum.

The *bactericidal action of an immune serum* is due to a specific thermostable[1] antibody (*bacteriolysin* or *bactericidin*) acting along with a normal non-specific constituent of serum (*complement*) which is thermolabile[2] (at 55° C.). The antibody apparently combines firmly with the antigen (the bacteria) and the complement then unites with the combined antigen and antibody. The antibody and complement have no independent combining affinity. It has thus been supposed that the antibody acts by "sensitising" the bacteria to the action of complement, and that the latter is the essential lytic agent.

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[1] "Thermostable" denotes the ability to withstand a certain degree of heat.

[2] "Thermolabile" indicates that a particular property is lost on exposure to a certain degree of heat.

Thus :

$$\begin{aligned}
&\text{Bacteria} + \left\{\begin{matrix}\text{Specific}\\ \text{antibody}\end{matrix}\right\} + \text{Complement} &&= \begin{matrix}\text{Bactericidal}\\ \text{effect.}\end{matrix}\\
&\text{Bacteria} + \left\{\begin{matrix}\text{Specific}\\ \text{antibody}\end{matrix}\right\} &&= \text{No effect.}\\
&\text{Bacteria} + \text{Complement} &&= \text{No effect.}
\end{aligned}$$

It may be noted here that complement is an exceedingly unstable substance and becomes quickly inactivated when serum is kept under ordinary conditions.

Owing to the original mistaken idea that the antibody acts merely as a combining link between the antigen and complement, the term "amboceptor" has been applied to it.

In vivo bacteriolysis can be demonstrated readily by *Pfeiffer's reaction* : a suspension of cholera vibrios is injected intraperitoneally in a guinea-pig along with an anticholera serum which is devoid of complement as a result of heating (*e.g.* at 55° C.) or keeping for some time, and if peritoneal fluid be drawn off with a hypodermic syringe at intervals within an hour, it is seen that the vibrios undergo progressive lysis and disappear from the fluid. In this case the complement of the blood plasma of the animal acts along with the antibody of the immune serum.

Bactericidal action by a normal serum, in many cases, is analogous to that produced by an immune serum, and is dependent on a natural antibody (*vide supra*) and complement. This mechanism, which is active mainly on Gram-negative bacteria, has been designated the "*α* lysin." Normal bactericidal effects towards various Gram-positive bacteria are independent of complement and are due to a thermostable (55° C.) principle "*β* lysin" the exact nature of which is still undetermined.

Haemolysis by a haemolytic antiserum is analogous to bacteriolysis—*i.e.* it is due to a specific thermostable antibody acting along with the normal complement.

Thus:

$$\text{Red Cells} + \left\{\begin{matrix}\text{Specific}\\ \text{antibody}\end{matrix}\right\} + \text{Complement} = \text{Haemolysis.}$$

$$\text{Red Cells} + \left\{\begin{matrix}\text{Specific}\\ \text{antibody}\end{matrix}\right\} \qquad = \text{No effect.}$$

$$\text{Red Cells} + \text{Complement} \qquad = \text{No effect.}$$

The phenomenon of haemolysis by serum can be demonstrated *in vitro* with blood suspensions and is easily visible with the naked eye, the blood becoming laked or transparent.

A suspension of red blood corpuscles in isotonic salt solution *plus* the antiserum which has been heated at 55° C. to annul complement (*i.e.* red cells+specific antibody *only*) serves as an indicator for the presence or absence of complement—*e.g.* in complement-fixation tests—and is spoken of as a *haemolytic system* (*vide* p. 43).

The *opsonic action of normal serum* is dependent on a non-specific thermolabile principle, the *normal opsonin*, which in some respects resembles complement (*v. supra*).

The increased *opsonic action of an antibacterial serum* is due to a specific thermostable antibody (*immune opsonin*) which can function independently of complement.

The opsonins combine with the bacteria, rendering them in some way susceptible to phagocytosis but without directly affecting their viability. Thus, if a serum is allowed to interact with organisms, which are then separated from it by centrifuging and washing with salt solution, they are still susceptible to phagocytosis by leucocytes though the serum has been removed and the leucocytes have also been freed from serum. The opsonin is apparently "absorbed" from the serum by the organisms and bound by them.

The *opsonic index* is a numerical expression of the opsonic power of the serum of a person for a given organism as compared with normal, and has been regarded as significant of the degree of resistance possessed by the person to the particular infection. The index can be estimated according to the following system: a preparation of leucocytes, the bacteria in question and the patient's serum are mixed, and, after a period of incubation, film preparations are made from the mixture and suitably stained; by counting under the microscope the number of bacteria phagocytosed by fifty, or preferably a larger number of, leucocytes, the average for one leucocyte can be calculated—the *phagocytic index.* A similar experiment is carried out, substituting the pooled serum of two or three normal persons, and the phagocytic index again estimated. The opsonic index is then calculated by dividing the phagocytic index of the serum in question by that of the normal serum. Thus unity is normal, and the opsonic power of the serum is greater or less than normal according as the index is greater or less than unity.[1]

It has been shown that the results of counting bacteria in a small number of leucocytes, *e.g.* 50, may not be a statistically accurate representation of the actual number phagocytosed.

Agglutination is the most easily observed effect of an antibacterial serum. If the serum is added *in vitro* to a uniform suspension of the particular organism and the mixture is incubated, the bacteria become aggregated in clumps and the suspension appears flocculent or granular, the clumps or floccules being easily visible to the naked eye. The phenomenon is attributed to a specific antibody designated *agglutinin.* The agglutinin does not affect the vitality of the bacteria. It also clumps dead bacteria in the same way as the living organisms. It is relatively thermostable—*e.g.* as compared with complement. Salts (electrolytes) are necessary for its action, and agglutination tests are usually carried out in a medium of physiological saline. The physics of bacterial agglutination has not yet been fully elucidated but depends apparently on a disturbance in the balance between the cohesive force of surface tension tending to aggregate the bacterial cells and the mutually repellent influence of the similar electrical charges carried by the cells. The electrolyte present, as well as the agglutinin, contributes to the physical changes involved in the process of agglutination. Agglutination tests are applied in diagnostic work, and for the identification of species and types (*vide* p. 28).

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[1] See Wright, *Technique of the Teat and Capillary Glass Tube*, London, 1921; *Studies in Immunity*, 1909.

As in the case of other antibody reactions, normal serum may contain agglutinins for various bacteria. In general, normal agglutination is quantitatively weak.

AGGLUTINATION REACTIONS IN RELATION TO ANTIGENIC STRUCTURE AND ANTIGENIC VARIATION.—Among motile bacterial species (e.g. *B. typhosus*) two different kinds of agglutinogen (*i.e.* the antigen which stimulates agglutinin production) can be recognised : *flagellar* (contained in the flagella) and *somatic* (in the body of the organism). The flagellar antigen is usually designated by the symbol H and the somatic by O. For these different types of antigen separate agglutinins are likewise produced also designated H and O, and the agglutination which results from the interaction of these antigens and agglutinins are described by the same symbols. Further, H-agglutination is of a "large-flake" type, *i.e.* large easily visible clumps, whereas O-agglutination is of the "small-flake" or granular type. Differential testing of H and O agglutinins can be carried out by varying the condition of the bacterial suspension. For H-agglutination a motile strain of the particular organism must of course be used and if the suspension is treated with formalin an almost pure H reaction is obtained since formalin interferes with O agglutination. Treatment of the suspension with alcohol, on the other hand, inactivates the H antigen and an alcoholised suspension is therefore a suitable reagent for testing O agglutination (*vide* p. 257). Another method is to use a non-motile variant of the organism. The H and O antigens differ in thermostability : thus the H antigen is labile at 80°–100° C. whereas the O antigen withstands 100° C.. A bacterial suspension which has been kept at 100° C. for about 20 minutes yields an almost pure O agglutination.

Some motile bacteria are *diphasic* as regards the constitution of their H antigens, e.g. *B. paratyphosus B*, and may occur in two different forms, one in which the H antigen is highly specific, the other in which this antigen shows characters common to related species or types. These *specific* and *non-specific* forms may be represented in a culture by different colonies so that if one colony is subcultured the " specific " phase is obtained, if another the " non-specific " phase, but such colonies may not show any morphological differences. Other species are *monophasic* as regards their H antigen, occurring only in the specific or in the non-specific form. A further variation may also be observed : the specific form may itself be diphasic : in one of these phases the H antigen has a different constitution from that of the other. These have been described as the α and β phases. It must be recognised that even a particular kind of antigen is frequently composite and consists of multiple components for each of which a separate agglutinin is produced on immunisation.

All these aspects of antigenic structure are well illustrated by the *Salmonella* group of bacteria, as is shown in the table on p. 450 which should be referred to.

In laboratory cultures of the typhoid-paratyphoid-dysentery bacilli two types of colony may be observed : (1) the normal smooth, round and transparent form—S (*smooth*) type, and (2) a rough, irregular and opaque variant—R (*rough*) type. The S type when suspended in 0·85 per cent. saline forms a stable suspension ; the R type tends to auto-agglutinate, though it may remain stable in weaker saline solutions (*e.g.* 0·2 per cent.). A similar variation with the development of R colonies is seen in other bacterial groups. These types are antigenically different ; thus, an antiserum for S may not agglutinate R and an antiserum for R may not agglutinate S.

The variation from S to R is associated with a change in the somatic antigen, the variant antigen being designated by the symbol Ø (or R) ; the H antigen is either unaltered or lost, the variant becoming non-flagellate. Among pathogenic bacteria this transformation from S⟶R is frequently associated with loss of virulence. Moreover, while the antigen characteristic of the S type is often highly specific, the R antigen may exhibit characters common to other, though related, species.

It has also been shown that the typhoid bacillus when freshly isolated possesses an additional somatic antigen which is associated with its virulence (as judged by injection of cultures in mice). When the organism is continuously cultivated artificially and loses virulence this antigen is no longer present. It has been designated the *Vi* antigen and can be detected by agglutination tests with an appropriate antiserum. Further reference to this subject is made later (p. 432). It seems likely that various organisms possess analogous "virulence" antigens and the capsular hapten of the pneumococcus has a somewhat similar rôle (*q.v.*).

These facts summarise briefly the variations in antigenic composition which have to be allowed for in the practical application of the agglutination phenomenon for diagnostic purposes. Further references to this question are made in later chapters. For more detailed information on the subject one of the text-books should be consulted.

The *precipitating action of an antibacterial serum* is analogous in many respects to agglutination and is spoken of as being due to an antibody called *precipitin.* For this test a suitable extract of the bacterial substance is required; this is exemplified in the precipitation test used for determining groups among the streptococci (*vide* p. 337).

Complement-fixation.—Complement plays an essential part in the bactericidal action of an immune serum, and is absorbed by the bacteria *plus* bactericidal immune body. Even in the absence of bactericidal action, an immune serum may contain antibodies which, along with antigen, fix or absorb complement; and a *complement-fixing antibody* is therefore spoken of.

Thus:

$$\left[\text{Antigen} + \begin{matrix}\text{Specific}\\ \text{antibody}\end{matrix}\right] \leftarrow \text{Complement.}$$

To test for this effect the haemolytic system is used as an indicator: if complement has been fixed, then on adding the haemolytic system no haemolysis will occur (*vide* p. 39).

Complement-fixation tests are employed in diagnosis, and occasionally for the identification of species in the same way as the agglutination reaction.

The various immune properties of an antibacterial serum have been described above in terms of multiple antibodies, each designated according to its effect on the antigen (bacteriolysin, agglutinin, opsonin, precipitin, complement-fixing antibody), but it is still an open question whether these reactions are actually due to separate principles in the serum or are merely different manifestations of the activity of a single antibody.

Antiviral sera.—The serum of a person or animal immunised against a filterable virus may exhibit properties which are analogous to those of an antibacterial serum. Thus, the virus is specifically inactivated by the serum, and this has been spoken of as due to a "viricidal" antibody, the term implying the killing of the virus. The actual effect of this antibody on the virus has not been fully determined. Specific precipitating and complement-fixing reactions have also been observed with antiviral sera. Where "elementary" or virus bodies can be separated and concentrated to form a suitable suspension, their specific agglutination by an antiserum can be demonstrated. This is well exemplified in the case of the elementary bodies of vaccinia (*vide* p. 619).

ANAPHYLAXIS AND HYPERSENSITIVENESS

Under certain conditions the parenteral injection of foreign protein leads to a specific sensitising effect so that the subsequent injection of the same substance may produce toxic and even fatal results.

Thus, if a guinea-pig is injected subcutaneously with horse serum (even an extremely small dose—*e.g.* 0·001–0·01 c.c.), and after an interval of ten days receives a larger second dose of the same serum (*e.g.* 0·2 c.c. intravenously or 5 c.c. subcutaneously), it may develop a sudden illness or *anaphylactic shock* in which the chief manifestation is spasmodic constriction of unstriped muscle, particularly that of the small bronchi. The serum may be quite non-toxic *per se* when given to an unsensitised animal even in large doses.

Substances that lead to anaphylaxis (*anaphylactogens*) are those which act as antigens in relation to immunity, and the phenomenon of anaphylaxis is regarded as due to the interaction of antigen and antibody (*precipitin*) in the tissues. Passive hypersensitiveness can also be conferred by injecting the serum of a sensitised animal into a normal animal.

The results of anaphylaxis depend on the mode of injection of the substance into the sensitised animal and the quantity introduced. Anaphylactic shock is more liable to occur and is more marked when the injection is intravenous or intraspinal, and when a large dose is given, than when the injection is subcutaneous or a small quantity is introduced. Thus, a dose which would produce shock if given at once, when introduced in small fractions may not lead to an anaphylactic shock and the animal is " desensitised " in this way. The state of hypersensitiveness, once developed, may persist for long periods. If, after the sensitising injection but before sensitiveness has developed, a second dose of the substance is given, the animal is protected for a time against a subsequent injection—i.e. *anti-anaphylaxis* has been developed.

Haptens (p. 35) may produce anaphylactic shock in specifically sensitised animals.

The nature of these phenomena has not been completely elucidated. Various theoretical explanations have been advanced on the basis of experimental data. For further details one of the larger works should be consulted. Serum anaphylaxis has, of course, become of practical importance in medicine in relation to serum therapy—for example, when it is necessary to give a second dose of a therapeutic serum after an interval, and especially by intravenous injection; the risk of anaphylactic shock can, in such cases, be obviated by desensitisation with very small doses of serum (*vide supra*). The human subject, however, is not so liable to anaphylaxis as certain animals.

Such hypersensitiveness can be tested for by injecting intracutaneously (*vide* p. 343) 0·1 c.c. of the serum. A positive result is denoted by the occurrence within thirty minutes of an urticarial reaction at the site of inoculation, which may develop a vesicle and may be surrounded by a wide erythematous zone. Hypersensitiveness can also be demonstrated by instillation of serum into the conjunctival sac, or by applying it to a small scarified area of skin.

Serum sickness or serum disease.—It should be noted that after a single dose of foreign serum (*e.g.* a therapeutic antiserum from the horse) given for the first time, in a considerable proportion of normal persons toxic effects may ensue. These occur after an interval of eight to twelve days, and may consist of fever, an erythematous or urticarial eruption, swelling of lymph glands and joints, albuminuria, etc., and there may be an inflammatory reaction at the site of the injection. Such symptoms have been regarded as due to a natural hypersensitiveness to the horse serum. It is still doubtful whether this condition is related to anaphylaxis or to the so-called "atopy" which is referred to later.

A single *intravenous* injection of foreign serum, especially in large amount, may in certain persons produce symptoms of shock. In the administration of therapeutic antisera such severe reactions can be avoided by a preliminary test for hypersensitiveness (*vide supra*). If this is detected, the required dose should be subdivided into fractions separately administered, starting with a small quantity.

Specific hypersensitiveness to the products of the infecting organism (*allergy of infection*) is a feature of various diseases in man and animals, and can be demonstrated by the general and local reaction manifested on injection of preparations from cultures of the particular organism—*e.g.* the tuberculin reaction (*vide* p. 402). In such cases the sensitiveness can also be elicited by simple cutaneous or intracutaneous tests. This form of hypersensitiveness differs from anaphylaxis in certain respects. Thus, it is doubtful whether such allergy is dependent on a serum antibody, and it has not been generally possible to transfer the sensitiveness to a normal individual by injection of serum.

Atopy.—In certain persons as a result of genetic factors, hypersensitiveness may occur towards a considerable variety of substances of protein nature, so that when the person is exposed to contact with the substance to which he is sensitive, toxic effects result —*e.g.* coryza, asthma, urticaria, gastro-intestinal disturbance, etc.. This form of sensitiveness has been designated *atopy*, and is responsible for such conditions as hay fever, asthma, etc.. Substances to which such sensitiveness can be attributed (*atopens*) are: plant pollens (as in hay fever), dandruff of animals (*e.g.* horse), proteins of various articles of food (*e.g.* shell fish), bacteria, moulds, etc.. Atopy can be tested for by cutaneous reactions with preparations of the particular *atopen*, as in testing infection-allergy. While atopy cannot be transferred in the same way as anaphylaxis, it has been found that when the serum of an atopic person is injected into the skin of a non-sensitive person, and after an interval the atopen is injected at the same site, an urticarial wheal results (Prausnitz-Küstner reaction). The serum therefore contains some active substance (designated *reagin*). This principle, however, cannot be definitely identified with antibodies.

Shwartzman phenomenon.—This reaction merits consideration in relation to the general subject of hypersensitiveness. It was observed by Shwartzman that, when a filtrate of *B. typhosus* culture had been injected into the skin of a rabbit and after 24 hours the same filtrate was injected intravenously, an intense reaction occurred at the site of the intradermal injection, viz. an area of haemorrhagic inflammation with subsequent necrosis. After a longer period had elapsed, *e.g.* 32 hours, intravenous injection might have no such effect. The reaction is not specific: thus, after the intradermal injection of *B. typhosus* filtrate, a *B. coli* filtrate injected intravenously may excite the reaction. The precise significance and the underlying biological mechanism of this phenomenon are still somewhat obscure.

PART II

Bacteriological Technique

MICROSCOPY ; CULTIVATION OF BACTERIA ; STAINING METHODS ; ANIMAL INOCULATION AND AUTOPSY ; IMMUNOLOGICAL AND SEROLOGICAL METHODS ; BACTERIOLOGICAL EXAMINATION OF WATER AND MILK ; TESTING OF ANTISEPTICS ; TESTING OF SURGICAL CATGUT FOR STERILITY ; COLLECTION AND FORWARDING OF SPECIMENS FOR BACTERIOLOGICAL EXAMINATION

CHAPTER III

THE USE OF THE MICROSCOPE IN BACTERIOLOGY

MICROSCOPY is of primary importance in bacteriology, and a suitable microscope and a knowledge of its use are essential to those engaged in bacteriological work. To obtain satisfactory results, the microscope should be used under the best possible conditions, and the worker must know the capabilities of the instrument.

The component parts of the microscope are as follows :—

The *eye-piece* fits into a graduated *draw-tube*, which in turn slides in the *body-tube*. The lower end of the tube is furnished with a *revolving nose-piece* (preferably " triple ") into which are screwed the *objectives*. The tube and objectives are moved up and down by means of a rack and pinion termed the *coarse adjustment*, while the fine movements necessary for accurate focussing are performed with the *fine adjustment*. The tube and adjustments are supported by an *upright* which is connected to the *foot* by means of a *hinged joint*. Attached to the upright near the joint is a platform called the *stage*, on which is placed the microscopical preparation to be examined. In the centre of the stage is an aperture through which the object can be illuminated from below. A *mechanical stage* may be fitted either to the stand or the fixed stage, so that the preparation can be moved about horizontally, thus ensuring steady and controlled movement. Below the stage is the *substage*, which should be furnished with a means of raising or lowering it—

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usually a rack and pinion or a spiral screw. The substage is fitted with a *substage condenser*, attached to which is the *iris diaphragm*. Fitted to the end of the *tail-piece* is a *mirror*, mounted on a gimbal fitting. The plane side of the mirror is employed when a condenser is used ; the concave side is used only in the absence of a condenser, and its focal length is such that light comes to a focus on the object examined.

The draw-tube should be adjusted to the *tube length* (*vide* p. 55) for which the objective is corrected ; for the majority of microscopes this is usually 160 mm., but as the revolving nose-piece has a length of 18 mm. the draw-tube should be extended only to the 142 mm. mark. Leitz objectives, however, are corrected for a tube length of 170 mm. It is essential that an objective should be used at its proper tube length, particularly the apochromatic objectives (*vide infra*), if the maximum resolution is to be obtained.

The working of the *fine adjustment* varies according to the make of the microscope, for different manufacturers have their own particular type of mechanism. The older forms were actuated by means of a milled head mounted on a pillar behind the coarse adjustment, which turned a screw with a fine thread. Present models have a milled head parallel to the coarse adjustment. The movements of the milled head raise or lower the tube in the same direction as the coarse adjustment. The milled head is graduated in $\frac{1}{100}$ths, and one division corresponds usually to a movement of the tube of 0·002 mm. The manner of securing a fine movement is by a system of levers, cams or cogwheels.

The fixed *stage* should be large enough to take a 4 in. Petri dish, and should be fitted with two clips. A *mechanical stage* is of great advantage, and is particularly useful when a large area of a microscopic preparation has to be searched, as in the examination of films of sputum for tubercle bacilli, or of blood for malaria parasites. An attachable mechanical stage works satisfactorily and can be obtained for almost any model. The " built-in " mechanical stage is more costly, but is recommended for routine bacteriological work, as it is steadier and not likely to get out of order.

The *substage* is an important part of the microscope and one to which frequently little attention is paid. The mechanism for raising the substage should be rigid and free from lateral movement. Where critical work has to be done with highly corrected objectives and condenser, centring screws should be fitted. The *condenser*, which is used for focussing light on the object to be examined, is usually of the two-lens Abbe type; but if apochromatic or semi-apochromatic objectives are used, a condenser of similar optical quality must be employed. The *iris dia phragm* is an important part of the substage, as it controls the angle of light which passes into the condenser. For example if the diaphragm is partially closed and a high numerical aperture objective is used, the definition will be much impaired.

Binocular microscopes.—Where much microscopic work has to be done and for routine examinations we recommend that the microscope should have a binocular body, as, by using both eyes, a considerable amount of eye strain and fatigue is avoided. In the binocular body the rays of light from the objective are divided by a half-silvered surface inclined at an angle of 45 degrees which permits one half of the light to pass vertically, while the remainder is reflected horizontally. Each half of the rays is directed into its appropriate eye-piece by means of prisms. The eye-piece sockets can be adjusted to the interocular distance of the observer, while one of the ocular tubes is adjustable to correct individual difference between the two eyes.

Inclined binocular microscopes are very suitable for routine use as the eye-pieces are inclined towards the observer and it is not necessary to tilt the stand as with the straight binocular or monocular bodies. Consequently the stage is kept horizontal and this is of particular advantage when dealing with wet films or using dark-ground illumination. (Similarly an inclined eye-piece fitting for a monocular tube may be obtained.)

Binocular microscopes have interchangeable monocular and binocular bodies, which are removable without disturbing the objectives, so that a monocular body can be used for photography, micrometry, etc..

OBJECTIVES AND EYE-PIECES

For general purposes, ordinary achromatic objectives are quite satisfactory, and are admirable for routine work and students' use. The quality of the present-day objective is extremely good, and for ordinary work the purchase of the more expensive types is not recommended. The most useful objectives are $\frac{2}{3}$-in. or 16 mm., $\frac{1}{6}$-in. or 4 mm., the $\frac{1}{12}$-in. (thus designated, but actually $\frac{1}{14}$-in.) or 2 mm. oil-immersion, and, for dark-ground illumination and blood work, the $\frac{1}{7}$-in. oil-immersion lens should also be added. These should be used in conjunction with a 10-magnification (10×) Huygens eye-piece. A 5× eye-piece is often supplied, and is sometimes employed for searching when a larger field is desired without altering the objective. It is not practicable to use the ordinary (Huygens) eye-piece above 12×, and even this magnification gives some distortion and haziness of outline. Higher magnifications may be obtained by the use of apochromatic objectives and compensating eye-pieces. With binocular microscopes 6× or 8× eye-pieces are sufficient, as, owing to the division of the rays, less light enters each eye-piece. With 10× eye-pieces the field is apt to be too dark when ordinary illuminants are used.

MAGNIFICATION

The objective works at a distance from the object somewhat less than its focal length. A real, inverted and enlarged image is formed in the upper part of the tube, and this real image is magnified further by the eye-piece. Thus, the total magnification is the product of the separate magnifications of the objective and of the eye-piece, and depends on three factors :—

(1) The focal length of the objective.
(2) The magnifying power of the eye-piece.
(3) The distance between the lens system of the objective and the image produced — the "*optical tube length.*"

In actual practice, however, when calculating the magnification, the "*mechanical tube length*" is used ; the diagram on p. 56 illustrates optical and mechanical tube lengths.

The "*optical tube length*"—AB—is the distance between the posterior principal plane of the lens system of the objective and the plane of the image which is in the upper part of the draw-tube.

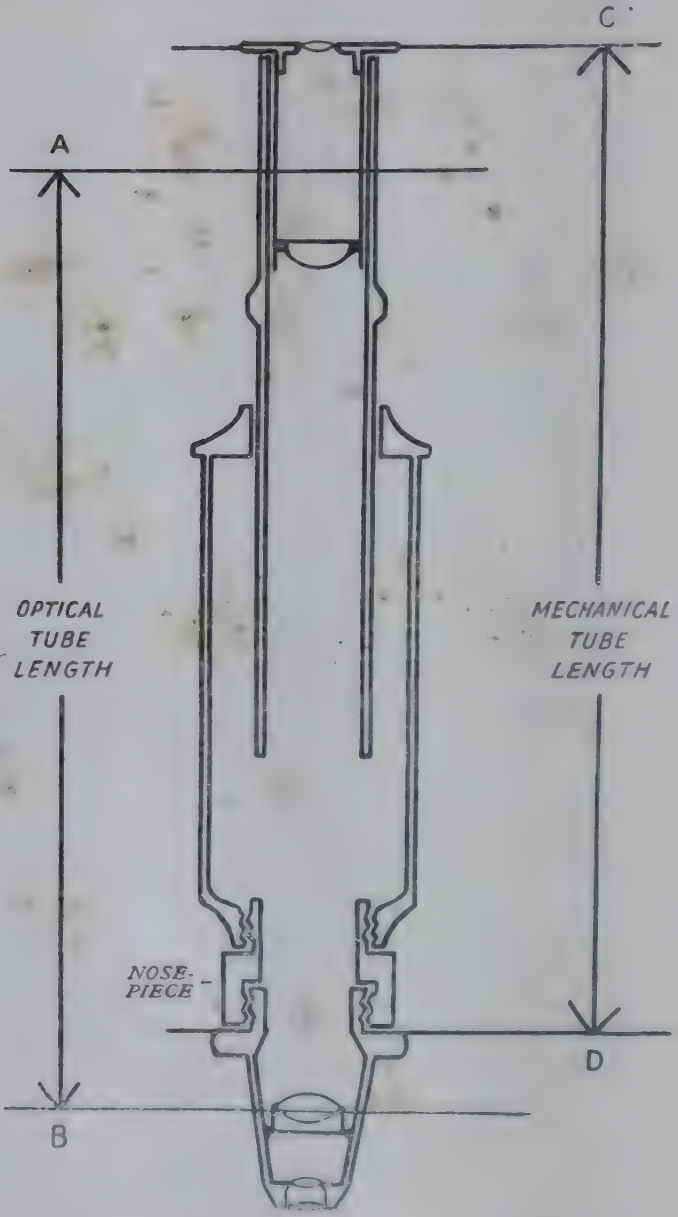
The "*mechanical tube length*"—CD—is the distance between the eye-lens of the eye-piece and the point where the objective fits into the lower end of the body-tube or nose-piece. In adjusting the draw-tube to a given tube length, it must be remembered that the draw-tube scale may read from the foot of the body-tube and does not include the nose-piece, the length of which is 18 mm. Allowance must be made for this—*e.g.* if the objective works at 160 mm. tube length, the scale of the draw-tube is set at 142 mm., which, with the length of the nose-piece, gives the required total length of 160 mm. In modern instruments the draw-tube scale includes the length of the nose-piece and the correct tube length is indicated on the draw-tube by an engraved ring. The tube length may easily be verified by measuring with a ruler from the bottom of the nose-piece to the upper end of the draw-tube.

When calculating the magnifying power of a given objective and eye-piece, the optical tube length may be taken for practical purposes as equal to the mechanical tube length.

It is emphasised that objectives are designed to work at a definite tube length and any variation from this distance may seriously impair the definition obtained, particularly when apochromatic and high-power achromatic objectives are used.

The initial magnifying power of the objective is first determined, and then multiplied by the magnifying power of the eye-piece, when the total magnification is obtained. The objective acts as a convex lens and its magnification can easily be calculated as follows :—

$$\text{magnification of objective} = \frac{\text{size of image}}{\text{size of object}}$$
$$= \frac{\text{distance of image from objective}}{\text{distance of object from objective}}$$


C
A
OPTICAL TUBE LENGTH
MECHANICAL TUBE LENGTH
NOSE-PIECE
D
B

The distance of the image from the objective is the " optical tube length " which is approximately equal to the " mechanical tube length," and this is determined from the draw-tube scale, as described above.

The distance between the object and the objective depends on the objective used, and this distance is adjusted by means of the coarse adjustment when the object is focussed. It may be taken as equal to the focal length of the objective. Suppose an object is examined with a 16 mm. ($\frac{2}{3}$-in.) objective and a 10 × eye-piece, the tube length being 160 mm.; the size of the image produced by the objective alone depends on the ratio of the tube length to the focal length of the objective—*i.e.* 160 mm. : 16 mm. (ten times); this real image is now magnified ten times by the 10 × eye-piece, making a total magnification of 100 diameters. If a 4 mm. ($\frac{1}{6}$-in.) objective is employed the distance of the image is the same (160 mm.), but the distance between objective and object is only 4 mm., hence the initial magnification of the objective is 160 : 4—*i.e.* 40. This is further magnified by the 10 × eye-piece to 400 diameters. Similarly a 2 mm. objective has an initial magnification of 80, and when used in conjunction with a 10 × eye-piece gives a total magnification of 800 diameters.

$$\text{Thus : total magnification} = \frac{\text{tube length}}{\text{focal length of objective}} \times \text{eye-piece magnification.}$$

To find the magnification of any system of objective and eye-piece, divide the tube length employed by the focal length of the objective; multiply this figure by the magnification of the eye-piece, and the total magnification is obtained.

It is thus seen that the magnification varies inversely as the focal length of the objective, the shorter the focal length the greater the magnification.

Makers now engrave the initial magnification of the objective on the objective mount, and as the eye-pieces are also designated by their magnifying power, the total magnification is easily and correctly determined, provided of course that the proper tube length is used.

The $\frac{1}{12}$-in. objective has, in reality, a shorter focal length than that by which it is designated, and gives a magnification of 100 diameters. When used in conjunction with a 10 × eye-piece the total magnification is 1000 diameters.

The magnification usually employed in bacteriological work is 800–1000 diameters.

The $\frac{1}{12}$-in. oil-immersion lens works very close to the cover-slip, and the intervening space between objective and cover-slip is filled with cedar-wood immersion oil.

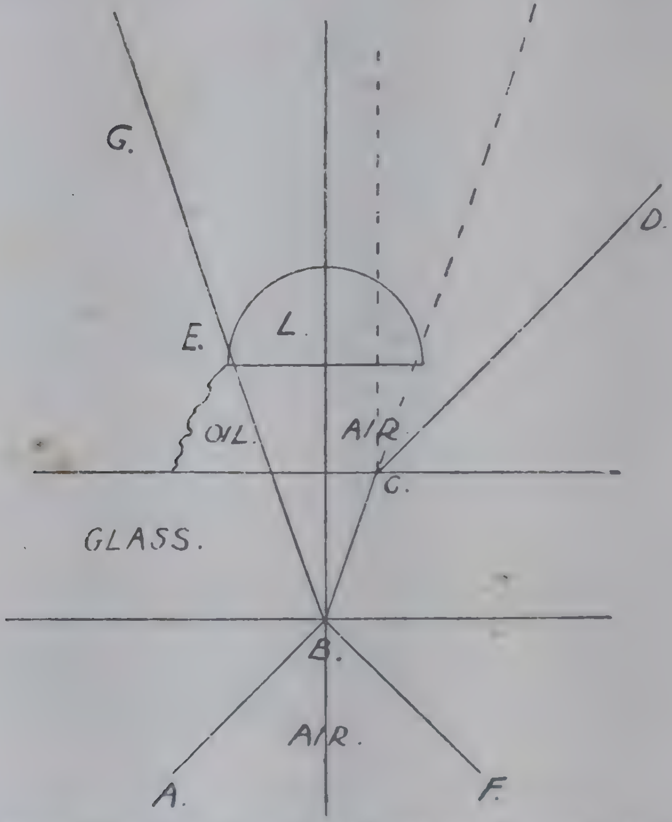


Diagram showing the paths of rays through (1) a dry lens (on right), and (2) an oil-immersion lens (on left) (*after* Spitta).

Note the refraction of the oblique ray ABCD in passing from the glass slide to air, as compared with the ray FBEG. L is the front lens of the objective.

The reason for this is that when an oblique ray of light emerges from a dense medium (glass) into a rare medium (air) it is refracted outwards –*i.e.* away from the normal (see diagram—ABCD). As the brightness of the image depends upon the light entering the objective, and the resolution (*vide infra*) depends on the effective aperture, this refraction of light diminishes not only the brightness but the clearness of the image. If, however, the space between objective and object is occupied by immersion oil, which has the same refractive index as glass, the rays of light do not undergo refraction and pass into the objective (see diagram—FBEG).

The high-power ($\frac{1}{6}$-in.) is a " dry " lens and must *not* be used with immersion oil. Oil must be used only with lenses specially computed to work with this fluid. Such objectives have " oil immersion " engraved on them.

NUMERICAL APERTURE

Objectives are rated not only by their focal length but also by their *Numerical Aperture* (N.A.). The numerical aperture may be defined simply as the ratio of the diameter of the lens to the focal length.[1] It is expressed mathematically as follows :—

$$\text{N.A.} = n \sin U$$

where n is the refractive index of the medium between object and objective (air, 1·0 ; cedar-wood immersion oil, approximately 1·5), and 2U the *angle of aperture*—*i.e.* the angle formed by the two extreme rays of light, which, starting from the centre point of the object, reach the eye of the observer (see diagram, p. 60).

That is, $\text{DAC} = 2\text{U}$

$$\text{BAC} = \text{U}$$

$$\sin \text{U} = \frac{\text{EF}}{\text{EA}}$$

It is thus seen that the numerical aperture, other things being equal, depends on EF, which is half the diameter of the lens. Lenses, therefore, may have equal focal lengths, but different numerical apertures depending on the diameter of the lens. It is to be noted, however, that the numerical aperture is not measured directly by the diameter of the lens, but is approximately the ratio of that diameter to the focal length.

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[1] The numerical aperture has been expressed in this manner to simplify description, but this is true only for objectives of long focal length, where EA is approximately equal to FA. With short-focus lenses of high numerical aperture this definition is not correct. The length EA is then much greater than the distance of the objective from the slide (FA).

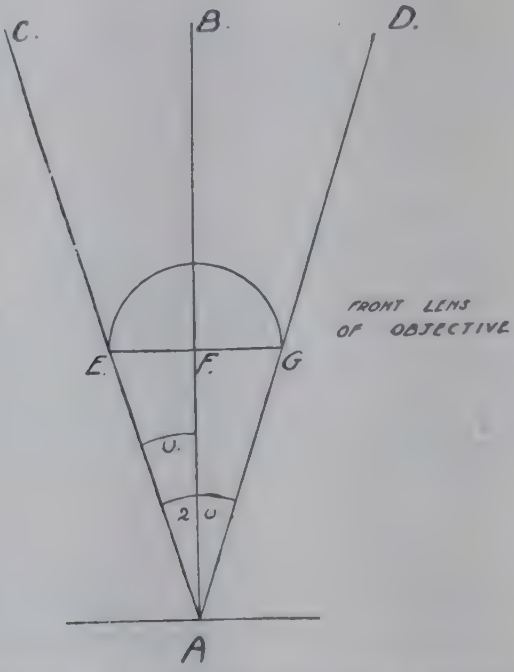

Diagram to illustrate numerical aperture.

The theoretical limit of the angle DAC is 180°—*i.e.* when the objective is actually on the object—and therefore the theoretical limit of U is 90°. The greatest possible N.A. of a dry lens cannot exceed 1, since the refractive index of air $(n)=1$, and $\text{Sin } 90°=1$. Actually the highest practical N.A. of a dry lens is 0·95. On the other hand, the introduction of oil between the objective and object gives n a value of 1·5. The highest possible value, therefore, of n Sin U for an oil-immersion objective is $1{\cdot}5 \times \text{Sin } 90°$—*i.e.* 1·5. In practice, however, the highest N.A. of an oil-immersion objective (attained in an apochromat) is 1·40.

The ordinary $\frac{1}{12}$-in. objective for bacteriological purposes has a N.A. of 1·30.

The essential qualities of an objective depend on its numerical aperture, and these are :—

(1) *brightness of image*, which, other things being equal, varies as the *square* of the N.A. ;
(2) *resolving power* and *defining power*, which vary directly as the N.A.

The *depth of focus*, while not dependent entirely on the N.A., varies in inverse proportion to it.

It is thus apparent why oil-immersion objectives give such good results—the N.A. being increased by the high refractive index of the oil. In general it may be said that in the case of two lenses of equal focal length the one with the higher N.A. is the better lens and is to be preferred. The 16 mm. or $\frac{2}{3}$-in. objective should have a minimum N.A. of 0·25 ; the 4 mm. or $\frac{1}{6}$-in. a minimum N.A. of 0·65 ; while the $\frac{1}{12}$-in. oil-immersion should have a N.A. of 1·30.

The *resolving power* (as apart from magnifying power) of a lens is its capacity to separate two adjacent points, and this property determines the amount of structural detail that can be observed microscopically. The limit of resolution is attained when the magnification reaches 1450 diameters. Theoretically, with axial illumination two points closer together than half the wave length of the light used cannot be resolved. It is not possible to attain this theoretical limit under visual working conditions, and in practice the limit is reached at about 0·00025 mm. (0·25μ).

Resolution, however, must not be confused with visibility, because it is possible to see "elementary bodies" (of virus diseases) as small as 0·074μ with ordinary white light and even smaller, 0·067μ, with green light (Coles). It should also be realised that the bodies observed have been stained and often treated with a mordant (*vide* Paschen's method, p. 225), so that the stained elementary body may be larger than the natural one, and thus brought within the limits of visibility. With ordinary microscopic methods, by using at the correct tube length an apochromatic objective of N.A. 1·40, and a high power compensating eye-piece, in conjunction with an oil-immersion condenser, the whole optical system and illuminant being carefully centred, stained particles of a diameter smaller than $0{\cdot}25\mu$ can be seen.

To illustrate the difference between visibility and resolution, if this printed page be placed a certain distance away (about 10 feet) it is possible to see that the print consists of a number of letters. It is not possible, however, to distinguish the form and shape of the actual letters as such at this distance. The letters are visible but their details cannot be resolved.

With ultra-violet light, which has a much shorter wave-length than visible light, greater resolution can be obtained. Advances with this method have been made by J. E. Barnard, using a specially constructed microscope, which requires special technical knowledge and skill for its use. As optical glass does not transmit short-wave ultra-violet light, special quartz lenses have to be employed. The wave-length employed is so short that objects illuminated by it are not seen by the naked eye, but must be recorded photographically. By this means Barnard has succeeded in photographing several of the filterable viruses (*vide* p. 659).

DEFINITION

This is the capacity of the objective to render the outline of an object distinct, and depends on the elimination of " spherical " and " chromatic " aberration.

Spherical aberration is caused by the periphery of the lens refracting more than the central portion. The peripheral rays, therefore, focus on the axis at a shorter distance from the lens than the central ones, with the result that the image is distorted.

Chromatic aberration is caused by the ray of white light being dispersed into its component colours as it is refracted through the lens, a spectrum being formed. The blue rays are refracted more, and come to a focus nearer the lens than the red rays. The different component colours do not come to the same focus and hence cannot blend to form white light. As a result, the image is fringed with colours and the outline is hazy.

Both chromatic and spherical aberration may be corrected by the combination of lenses of different dispersive power—*e.g.* convergent convex lenses of crown glass having low dispersive power, and divergent concave lenses of flint glass having high dispersive power. By this means two of the spectrum colours are combined, and the ordinary achromatic objective is constructed in this manner.

APOCHROMATIC OBJECTIVES

While achromatic objectives fulfil all ordinary purposes, they are not sufficiently corrected for critical work, such as photography and resolution of minute objects, for which apochromatic objectives must be employed. These represent the highest degree of optical perfection, and are, in consequence, very expensive. Apochromats surpass all others in the matter of colour correction, and the essential factor in their construction is the use of the mineral *fluorite*. Fluorite possesses the following valuable optical properties :—

(1) high degree of transparency ; (2) low refractive index ; (3) extremely small dispersion.

As a result of the use of fluorite at least three colours may be united, thus eliminating the secondary spectrum. This endows the objectives with a brilliance and " crispness " of image not attainable with ordinary lenses, and enables the maximum resolving power to be obtained.

Apochromatic objectives must be used only in conjunction with " compensating " eye-pieces, and care must be taken to adjust the tube length carefully and to employ a highly corrected and properly centred condenser.

A series of objectives containing a certain amount of fluorite, and which are intermediate in quality between the apochromatic and achromatic objectives, has been introduced by several makers. They are known as " semi-apochromatic " or " fluorite " lenses, and some of them have a performance approaching that of the apochromatic objectives. The oil-immersion lenses of this series are very useful for dark-ground illumination.

The student or beginner in microscopy is advised not to purchase apochromatic or fluorite lenses, as the present-day achromatic or ordinary objectives have been brought to such a pitch of excellence that all routine examinations and much research work can be done with them.

CARE OF THE MICROSCOPE

The microscope is an instrument of precision, and care must be taken to preserve its accuracy. The instrument should be kept at a uniform temperature and not exposed to sunlight or any source of heat. When not in use it must be protected from dust under a cover or in its box. Failing these, it should be covered with a clean duster or cloth. The microscope should be cleaned at intervals and its working surfaces very lightly smeared with soft paraffin (vaseline). With binocular microscopes dust may collect on the surfaces of the prisms. This may be removed by passing a soft camel-hair brush down the eye-piece tubes after removing the eye-pieces. On no account must the prism case be opened and the prisms removed, as this will completely alter the optical alignment and necessitate the return of the instrument to the maker before it can be used again.

If the microscope has to be moved, it should be lifted by the upright limb and not held by the body tube.

The oil-immersion objective must be cleaned each day after use by wiping the front lens with a well-washed silk or cotton handkerchief. Alternatively a fine tissue paper known as "lens paper" may be used. which is very suitable for the purpose. Oil remaining on the lens-front dries and becomes sticky; later it hardens and is then difficult to remove. Canada balsam accidentally present on the lens from a mounted microscopic specimen may also dry hard in the same way. When cleaning the objective *do not use alcohol*, as the cement that unites the component lenses is soluble in alcohol, and in consequence the lens systems may become disorganised and the objective spoiled. Benzol or xylol must be used to remove dried oil, and if the oil is hard, repeated applications on a soft cloth are necessary.

Dry objectives—*e.g.* $\frac{2}{3}$-in. and $\frac{1}{6}$-in.—are cleaned with a piece of well-washed silk or fine cotton, or lens paper. If any oil or Canada balsam is accidentally present on the front lens it must be removed with a soft cloth moistened in benzol or xylol and the lens quickly dried with a soft cloth. On no account must the component parts of an objective be unscrewed.

DIRECTIONS FOR USING THE MICROSCOPE WITH CONDENSER AND OIL-IMMERSION LENS

Before commencing to examine a specimen special attention must be paid to the following :—

(1) The objectives and eye-piece must be clean.

(2) The draw-tube must be adjusted to the correct length.

(3) The plane side of the mirror must be used.

(4) The condenser must be properly fitted into the substage, so that it can be racked up practically flush with the stage. In microscopes where the condenser is inserted from below into a sleeve fitting, the condenser is often not properly pushed into place and cannot be racked up sufficiently high for its focus to be in the same horizontal plane as the specimen.

For bacteriological work it is recommended that artificial light always be used. A 40 or 60 watt gas-filled electric lamp with a bulb of opal glass is highly suitable. It is convenient to have some form of microscope lamp which partially encloses the bulb, so that no glare reaches the eyes. It is not advisable to use the microscope at a window, as the daylight entering the eyes renders the vision less acute. A suitable arrangement is to use the microscope on a small table at one side of the room so that the observer's back is towards the window.

With binocular microscopes the amount of light reaching each tube is only half that of a monocular instrument, while some light is absorbed by the glass prisms. With high magnifications, therefore, the field may not be as brightly illuminated as would be wished. It is convenient, therefore, to "over-run" the lamp by using a bulb of lower voltage than that of the electric supply, *e.g.* a 200 volt bulb on a 230 volt circuit or a 150 volt bulb on a 200 volt circuit. The intensity of light is very much increased and satisfactory illumination secured. The amount of light may be controlled, and the life of the bulb prolonged by using a sliding resistance of 300 ohms to carry 1 amp. in series with the lamp. With low powers the intensity of light is dimmed by the resistance while for higher powers the full intensity is used.

When examining an object, the manipulations of the microscope should be carried out in the following order :—

(1) Set up the microscope, place the object on the stage, and adjust the plane side of the mirror to the illuminant so that the light is reflected into the condenser.

(2) Focus the specimen with the low-power objective, using the coarse adjustment.

(3) Manipulate the mirror until the image of the illuminant is seen in the centre of the field ; then lower the condenser so that the whole field is evenly illuminated.

It is essential, particularly when examining tissues, to use the low-power first, in order to locate organisms and observe the tissue changes. A suitable field having been obtained, the slide must be kept in place by means of the right-hand clip if a mechanical stage is not used.

(4) Rack up the objective a short distance and place a drop of cedar-wood immersion oil on the portion of the specimen immediately below the objective.

(5) Raise the condenser so that its upper surface is practically level with the stage, and make sure that the iris diaphragm is widely open.

(6) Rotate the nose-piece until the oil-immersion lens is in position.

(7) With the eye at the level of the stage, lower the objective by means of the coarse adjustment *until the lens is seen just to touch the oil.*

(8) Apply the eye to the microscope and observe if the field is well illuminated. If not, adjust the mirror until maximum illumination is secured.

(9) *Carefully* focus down, using the *coarse* adjustment, and when the object has come into view, use the fine adjustment to secure sharp definition. It is often necessary at this stage to raise or lower the condenser very slightly so that the optimum illumination is secured.

When the object is sharply focussed and the condenser is so adjusted that the image of the illuminant is seen in the field, the condition is termed *critical illumination.*

When using a binocular microscope the same directions should be observed, but, in addition, the eye-pieces should be adjusted to the correct interocular distance of the observer when the specimen is focussed with the low-power objective (*vide* direction No. 2).

When the observer has to examine a specimen for any length of time, as when searching for bacteria, he must adopt a comfortable position. Both forearms should rest on the table, and if there is no mechanical stage the slide is moved with the left hand while the right hand manipulates the fine adjustment.

EXAMINATION OF LIVING UNSTAINED ORGANISMS

In the case of bacteria, "hanging-drop" preparations are frequently used for this purpose, and a glass slide having a circular concavity in the centre is employed.

There should be no difficulty in observing a satisfactory specimen if the following procedure is adopted :—

(1) By means of a match dipped in vaseline, a ring or square (according to the shape and size of the cover-slip) is outlined round the concavity.

(2) With a platinum loop (*vide* p. 163) place a drop of fluid containing the organisms on a cover-slip laid on the bench.

For this purpose a fluid culture is used or the condensation fluid of a slope culture (*vide* p. 161). A further alternative is to emulsify a small amount of culture from the surface of a solid medium in a drop of broth or normal saline, taking care that the emulsion is not too dense.

(3) Invert the slide over the cover-slip, allowing the glass to adhere to the vaseline, and quickly turn round the slide so that the cover-slip is uppermost. The drop should then be " hanging " from the cover-slip in the centre of the concavity.

(4) Place the slide on the microscope, rack down the condenser slightly and partially close the iris diaphragm. (Excessive illumination renders the organisms invisible.)

(5) With the low-power, focus the edge of the drop so that it appears across the centre of the field.

(6) Turn the high-power ($\frac{1}{6}$-in. or 4 mm.) lens into position and focus the edge of the drop. Obtain the best illumination by lowering or raising the condenser, and secure sharp definition by reducing the aperture of the diaphragm.

Instead of employing a hanging-drop preparation, a film of the fluid between an ordinary slide and cover-slip may be used, but in this case the edge of the cover-slip should be sealed with vaseline to prevent evaporation of the fluid.

Motility of organisms can be detected in this way and their shape, approximate size and general structure can be observed. *It is advisable to use the high-power dry lens* and not the oil-immersion objective. Owing to the viscosity of the oil, the cover-slip is apt to move during focussing, and currents are thus caused in the fluid, which produce an appearance of motility in the organisms.

It is essential to distinguish between true motility, where the organism changes its position in the field, and Brownian movement, which is an oscillatory movement possessed by all small bodies (whether living or not) suspended in fluid (p. 9).

A *warm stage* is very convenient when examining fresh unstained preparations for amoebae and other protozoa. There are several types of warm stage available, some of which consist of a thin, flat metal box filled with hot water, or through which warm water can circulate, and having an aperture in the centre by which the light passes to the preparation. Other forms are electrically heated and have an automatic temperature control. The warm stage keeps the preparation at body temperature, and enables the movement of organisms to be studied, as these movements may cease if the material is kept for any length of time at room temperature.

A simple warm stage may easily be improvised from a sheet of thin copper (18-gauge) shaped like the letter " T," with the long arm 5–6 in. in length. The top of the " T " is the size of a microscope slide (3 in. × 1 in.) and in the centre is an aperture ½ in. in diameter. The copper " T " is placed on the microscope stage with the long arm projecting forward, and the aperture over the condenser. The preparation is placed on the copper strip and secured by the stage clips. The projecting part of the " T " is warmed by means of a small Bunsen flame or spirit lamp. Care must be taken that the preparation is not overheated.

DARK-GROUND ILLUMINATION

This method renders visible delicate organisms, such as the spirochaete of syphilis, which cannot be seen in unstained preparations with an ordinary microscope.

By means of a special condenser the specimen is illuminated by oblique light only. The rays do not enter the tube of the microscope, and, in consequence, do not reach the eye of the observer unless they are "scattered" by objects (*e.g.* bacteria) of different refractive index from the medium in which they are suspended. As a result, the organisms appear brightly illuminated on a dark background.

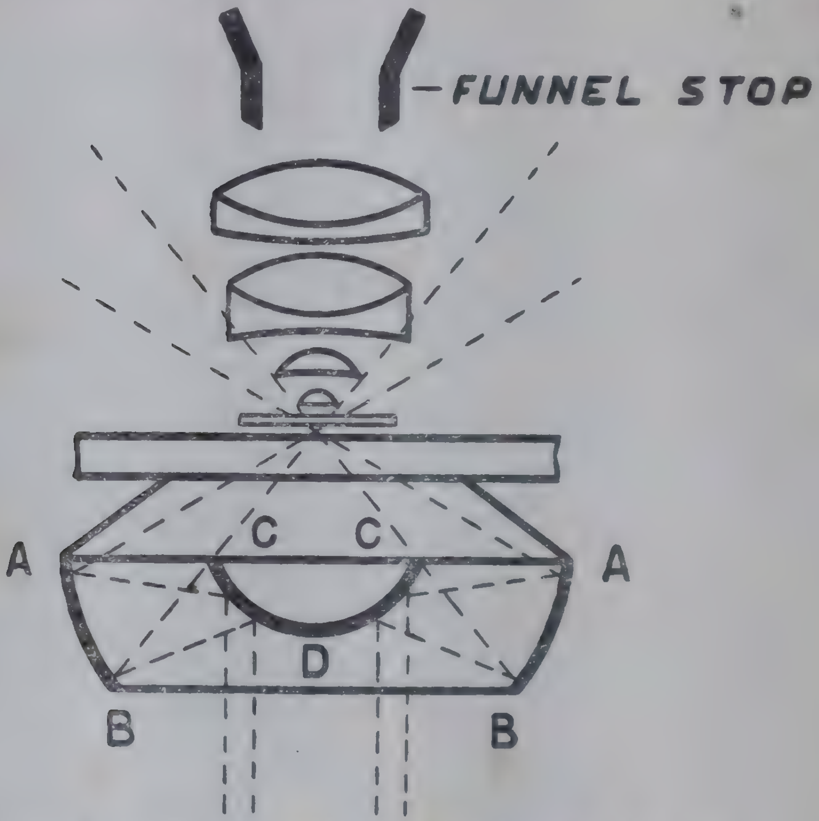

Diagram showing the paths of rays through the condenser and a $\frac{1}{12}$-in. oil-immersion lens fitted with a funnel stop. AB and CDC are reflecting surfaces. The surface at CC is opaque. (*After* E. Leitz.)

Three requisites are necessary for adapting a microscope for dark-ground illumination :—

(1) A "dark-ground" condenser.
(2) A suitable illuminant of sufficient intensity.
(3) A stop which reduces the numerical aperture of the objective to less than 1·0, *if the ordinary oil-immersion lens is used.*

The Condenser.—A special condenser must be employed and may be of the paraboloid or of the concentric spherical reflecting type. The latter is recommended. The function of the special condenser is to focus the light on the object, the paths of the rays being such that no direct light passes into the front of the lens. The illustration shows the paths of rays through the concentric reflecting condenser. The condenser should be furnished with a centring device. It must be emphasised here that success with dark-ground illumination depends on the accurate centring of the condenser.

The Illuminant.—A lamp of sufficiently powerful intensity should be employed.

If *direct current* only is available, the small arc lamp or the "pointolite" lamp (a proprietary name applied to a lamp consisting of two tungsten electrodes in a vacuum across which an arc is struck) should be used. The arc lamp is provided with a condensing lens and gives a higher intensity of light, but requires constant attention to keep the width of the arc adjusted as the carbons burn away. Arc lamps with a clockwork feed may be used to obviate this difficulty, but they are apt to be unreliable. The pointolite lamp which is adapted primarily for direct current requires a special resistance, and, in addition, an enclosed chamber with a condensing lens has also to be provided. On the other hand, the lamp requires no further attention, and gives a constant light for any length of time. The "pointolite" is more convenient to use than the arc lamp.

Alternating current is now rapidly becoming universal, and small filament lamps of high intensity worked through a transformer are inexpensive, economical and satisfactory. The bulbs are gas-filled and have a short, thick and tightly wound spiral filament. They take 4–5 amperes at 6 volts.

A powerful and intense source of light is the ribbon filament lamp. It consists of a broad flat ribbon of tungsten between two stout electrodes. It consumes 18 amperes at 6 volts and must be worked through a transformer. This lamp, which is also specially suitable for photomicrography, is recommended where much dark-ground illumination work has to be carried out.

A ventilated enclosed chamber with a condensing lens is necessary with both types of bulbs, and complete lamps are obtainable from several makers.

A pointolite lamp for alternating current is available, but is not so satisfactory as the direct current lamp. It is, in addition, more expensive than the coiled filament lamp and not so efficient as the ribbon filament lamp.

The Funnel Stop.—When the objectives employed for dark-ground illumination have a numerical aperture of more than 1·0 (as in the case of ordinary oil-immersion lenses), a special stop to reduce the N.A. to less than 1·0 must be employed. This consists of a small funnel-shaped piece of metal or vulcanite which fits into the objective behind the back lens. It is advisable to procure the stop from the maker of the lens employed. The stop is easily inserted and removed, and the objective can at once be converted for ordinary use.

Alternatively an *objective adapter*, with a small iris diaphragm, may be used. The front part of the oil-immersion objective is removed and screwed to the adapter, which then takes the place of the objective on the nose-piece. The numerical aperture of the objective may be reduced as desired by manipulating the iris diaphragm in the adapter. Some makers incorporate an iris diaphragm in the mount of the objective itself so that it can be used for bright or dark-ground illumination without further alteration.

Certain manufacturers have introduced for dark-ground illumination with bicentric condensers, special oil-immersion fluorite objectives, which are used without a funnel stop. These are $\frac{1}{12}$-in. N.A. 1·15, and $\frac{1}{7}$-in. N.A. 0·95. The latter lens can be recommended for routine dark-ground observation, and in addition is very useful for histological work in place of the $\frac{1}{6}$-in.

The Preparation.—The preparation should be as thin as possible in order to secure a satisfactory dark background, and so that the moving objects shall, as far as possible, be in one plane. A preparation which is too thick greatly diminishes the contrast in the dark field, and in order to obtain satisfactory contrast the objective has to be stopped down considerably, thus diminishing its resolving power. The preparation should not be too dense, otherwise there is an excessive number of particles which " scatter " the light. This causes lack of contrast even to a greater degree than a thick preparation. Some manufacturers supply special cells for dark-ground work so that when the cover-slip is placed on the slide the preparation has a definite and uniform thickness.

The thickness of the slide employed is important. The slides should be 1·0–1·1 mm. thick, and when a suitable supply has been obtained they should be used only for dark-ground work. They should be thoroughly clean and free from grease. The object to be examined must be at the focus of the condenser, the focal length of which is about 1·2 mm. If, therefore, too thick a slide is used, the focus of the condenser will be below the specimen and poor illumination will result; if the slide is too thin, the distance between the condenser and slide is such that a large amount of oil must be employed to make contact.

METHOD OF USING DARK-GROUND ILLUMINATION WITH THE OIL-IMMERSION OBJECTIVE

The microscope with special condenser and with the N.A. of the objective reduced by a funnel stop or iris diaphragm adapter is placed in front of the illuminant. It is advisable to have the microscope in the upright position and not inclined, to avoid running of the oil. The condensing lens of the lamp is adjusted so that a slightly converging beam of light is obtained. With the plane side of the mirror the light is directed into the dark-ground condenser. Using the low-power ($\frac{2}{3}$-in. or 16 mm.) objective, focus the surface of the condenser so that the engraved concentric rings on the surface come into view. These rings show the centre of the condenser, and if the condenser is out of centre adjust the centring screws so that the rings become concentric with the edge of the field.

Should the condenser have no engraved rings the centring may be accomplished as follows :—

A slide preparation is placed on the stage and oil contact between it and the condenser established. The preparation is focussed with the ⅔-in. objective, and, particularly if an arc or ribbon filament lamp be used and the mirror properly adjusted, a bright ring of light is noticed in the field. Focus the condenser up or down so that the ring of light contracts to the smallest bright spot obtainable. If this spot of light is not in the centre of the field the centring screws of the condenser must be altered accordingly.

The accurate centring of the condenser is of the utmost importance, and the time spent in this manipulation will be amply rewarded by the brilliant illumination obtained. The preparation to be examined must be covered with a No. 1 cover-slip, and it is advisable to ring round the cover-slip with vaseline to prevent evaporation. Place a large drop of immersion oil upon the under surface of the slide and also on the upper lens of the condenser, and a similar drop on the cover-slip. Place the slide on the microscope stage, taking care that the upper surface of the condenser is well below the slide. Rack up the condenser until oil-contact is made between the whole surface of the upper lens of the condenser and the slide ; then bring the oil-immersion lens into position so that it touches the oil on the cover-slip. Now carefully focus the specimen. A slight adjustment of the condenser, up or down, may be necessary, and some manipulation of the mirror may also be required. After a little practice an evenly illuminated field with an intensely dark background and brilliantly lit objects may be obtained with a minimum of trouble.

Where much dark-ground examination has to be done, it is recommended that a microscope be reserved solely for this work and kept ready with the illuminant in position, so that it is always available for immediate use. It is convenient to have the lamp and microscope fixed to a board for this purpose. The microscope, when not in use, should be covered to exclude dust.

After use the condenser and objective should be carefully wiped free from oil. Discarded preparations should be dropped into a covered jar of benzol kept for the purpose. When a sufficient quantity has been accumulated it will be found that they can easily be cleaned, as the oil and vaseline have been dissolved off by the benzol.

DARK-GROUND ILLUMINATION WITH LOW AND MEDIUM POWER LENSES

Dark-ground illumination is easily obtained with a low-power lens whose numerical aperture does not exceed 0·3, *e.g.* the ⅔-in. objective, by placing a central patch or stop below the Abbe condenser. Most manufacturers supply a set of stops which fit into the ring below the iris diaphragm. Alternatively, a circle of glass with a central patch of black gummed paper about 10–12 mm. in diameter may be used. The ordinary source of illumination is quite sufficient. Such dark-ground illumination may be used for observing slide-agglutination and for cells, casts, etc., in urinary deposits. With the higher power dry lenses, however, it is not so easy to secure satisfactory dark-ground illumination unless special condensers are used. Most microscope manufacturers make dry dark-ground condensers to work with ⅙-in. objectives up to numerical apertures of 0·85, but these are expensive and usually require a high intensity lamp to work satisfactorily. The results, however, are very beautiful.

Where the N.A. of the objective does not exceed 0·65, dark-ground illumination can be secured with an " achromatic " or " aplanatic " condenser (not Abbe condenser), an expanding iris or suitably large central stop being used. As such condensers are suitable for ordinary microscopy it is possible to change over from direct transmitted light to dark-ground illumination, without removing the condenser, by merely inserting the stop. A high intensity illuminant is, however, necessary. An intermediate objective adapter with iris diaphragm (p. 72) is often of value in reducing the N.A. of the ⅙-in. objective sufficiently to obtain a uniform dark field.

MICROMETRY

In bacteriological work the unit of measurement is 0·001 mm. designated a *micron* or μ. The measurement of microscopic objects is accomplished by means of the stage micrometer in conjunction with a micrometer eye-piece. The stage micrometer consists of a 3×1 in. slide on which is a millimetre scale graduated in hundredths of a millimetre. This scale is either engraved or made by a photographic process. The micrometer eye-piece consists of a special eye-piece in which a graduated scale, mounted on the diaphragm, can be focussed by means of the movable eye-lens.

When measurements are to be made the micrometer eye-piece is inserted into the draw-tube, the tube length being accurately noted, and the rulings on the stage micrometer focussed by the appropriate objective according to the size of the object to be measured. The number of divisions on the eye-piece scale corresponding to a definite number of divisions of the millimetre stage scale is determined. The stage micrometer is removed, and the object to be measured is next focussed. The number of divisions of the eye-piece scale which just cover the object are noted.

The millimetre value of each division of the eye-piece scale depends on the objective used and the tube length employed, and is usually determined each time a measurement is taken. Sometimes it is advisable to increase or diminish the draw-tube length so that the stage and eye-piece scales coincide or bear a geometric relation to each other—*e.g.* 1 division of the former to 10 of the latter.

Example : Using a $\frac{1}{12}$-in. objective and a 6 × micrometer eye-piece at 165 mm. tube length, it was found that 100 divisions on the eye-piece scale exactly covered 11 divisions of the stage micrometer. Each division of the stage micrometer is $\frac{1}{100}$ mm.

100 eye-piece divisions = 11 stage divisions = ·11 mm.
1 eye-piece division = ·0011 mm. = $1{\cdot}1\mu$.
1 eye-piece division, therefore, with the given objective, eye-piece and tube length = $1{\cdot}1\mu$.

The stage micrometer was removed and a stained slide of blood showing malaria crescents was substituted. The diameter of a red blood corpuscle covered 7 divisions of the eye-piece scale—*i.e.* $7{\cdot}7\mu$. A polymorph leucocyte covered 11 divisions, while the length of a malaria crescent was equal to 10 divisions, showing the sizes of these objects to be $12{\cdot}1\mu$ and 11μ respectively.

If the draw-tube is so adjusted that 1 division of the stage micrometer equals 10 of the eye-piece scale, then each division of the latter corresponds to 1μ.

Photographic Method of Micrometry.—A more accurate method is to photograph a film of the organisms or cells under a high magnification. Without disturbing the microscope or camera, the slide is removed from the microscope stage and the stage micrometer substituted. A photograph of the stage micrometer is then taken at exactly the same magnification. By means of a pair of fine dividers the length of the organism on the print is taken, and its exact measurement found by applying this distance to the micrometer print.

For brief notes on *Fluorescence Microscopy* and the *Electron Microscope* see **Appendix** (pp. 658 and 659).

CHAPTER IV

CULTIVATION OF MICRO-ORGANISMS

Only in exceptional cases can the identity of a bacterium be established by its morphological characters (p. 27). It is essential to obtain a *culture* by growing the organism in an artificial *medium*, and if more than one species or type are present these require to be carefully separated or isolated in *pure culture*. In this process there are three distinct operations:—

(1) The preparation of suitable culture media.

(2) The removal of other organisms from the medium, glass-ware, etc., by sterilisation. Bacteria are ubiquitous, and are present in the material and on the articles used for making media. These contaminating organisms must be destroyed so that the culture medium is sterile.

(3) The cultivation of the organism and its isolation from others present in the material to be examined. It is only occasionally that organisms can be grown directly from the body in pure culture.

CONTAINERS FOR MEDIUM AND CULTURES

In the past, flasks and test-tubes, stoppered with cotton-wool, have been universally employed as containers for medium and cultures. Recently, however, improved methods for the distribution and storage of culture media have been introduced, in which screw-capped bottles of varying capacity and shape are used for these purposes and substituted for the original types of container. Thus, medium can be distributed and preserved in hermetically sealed bottles on the same principle as the canning of foodstuffs. The designation "bottled" has been applied to media preserved in this way. These methods are particularly valuable in large laboratories where culture media are prepared in quantity for distribution.

In the following pages the original methods are described, as in previous editions of the book, but the use of screw-capped bottles and bottled media is specially dealt with for the guidance of laboratory workers, as apart from students who may be more interested in the ordinary methods applicable to the preparation and use of the simpler culture media.

PREPARATION OF GLASS-WARE

Washing and Cleansing.—New glass-ware requires special attention because of the resistant spores which are present in the straw and other packing material. Thorough mechanical cleansing with soap and hot water and the aid of a brush is not sufficient; neither are the spores always killed in the hot-air oven (*vide infra*). It is advisable to boil new glass for half an hour in 5 per cent. soft-soap solution. Some of the soap powders on the market cause clouding of the glass and in consequence should not be used. Glass containers with discarded cultures are usually placed in 3 per cent. lysol immediately after use, but those containing tubercle bacilli or spore-bearing pathogenic organisms, such as *B. anthracis* or *B. tetani*, must be autoclaved (p. 84). The discarded cultures and their containers are then boiled in 5 per cent. soft-soap solution for fifteen minutes in a covered boiler. The glass-ware is cleansed with a test-tube brush (or other suitable brush) and washed in running water to remove the soap. If the tap water is very "hard" and contains a considerable amount of calcium salts, rinsing in distilled water is necessary. The glass-ware is then allowed to drain and dry. See also **Appendix.**

Neutralisation of Glass-Ware.—New glass-ware, especially the cheaper varieties, gives off free alkali, and this may be sufficient to interfere with the growth of certain organisms. Where such slight changes of reaction are of importance, the glass-ware should be placed in 1 per cent. commercial hydrochloric acid for several hours, thereafter well washed in tap water, and finally in distilled water.

Screw-capped bottles (described later) are subjected to a special cleansing process by the makers whereby surface alkali is removed, and the treatment described above is not necessary. The bottles may be used, without further treatment, as received from the manufacturers.

METHODS OF STERILISATION

Various methods are employed for the destruction of bacteria, according to the nature of the article to be sterilised; and each method has its own particular use, with well-defined limitations. The usual methods of sterilising are either (*a*) destruction of the organisms by some form of heat or by chemical antiseptics, or (*b*) mechanical removal by filtration.

Chemical methods of sterilisation are not employed in the preparation of culture media, as the presence of the chemical which destroys the contaminating organisms will either kill or prevent the growth of the bacteria artificially introduced into the medium.

For the sterilisation of most culture media and apparatus, heat is applied in some appropriate form, depending on the nature of the object to be sterilised.

STERILISATION BY HEAT

Objects may be sterilised by heat in two forms—dry heat (Bunsen flame or hot air) and moist heat (steam or hot water). Sterilisation by dry heat requires a much higher temperature, or a much longer time at the same temperature, than in the case of moist heat.

To ensure complete sterilisation, all forms of bacterial life must be destroyed, *and the time of sterilisation is that necessary to kill the most resistant forms.* Spores are extremely resistant to all methods of destruction, whether by means of heat or chemicals, and whereas the vegetative phase of an organism such as *B. anthracis* is killed by moist heat at 100° C. in a few seconds, the spores resist boiling for five minutes. Spores of some saprophytic bacteria may survive boiling for periods up to $1\frac{1}{2}$ hours. The time for effective sterilisation, therefore, by any method is that which ensures complete destruction of all spores.

STERILISATION BY DRY HEAT

(1) *Red Heat.*—Inoculating wires or needles, points of forceps, and searing spatulas are sterilised by this method, the heat from an ordinary Bunsen burner being utilised.

(2) *Hot-Air Oven.*—In its original form this consists of a chamber having double walls between which hot air passes from a Bunsen burner. The oven may, however, be heated electrically. It is desirable to have an automatic device which keeps the temperature constant at any predetermined level. A temperature of 160° C. for one hour or 180° C. for half-an-hour is necessary for complete destruction of bacterial spores. (It is obvious that such a temperature cannot be utilised for the sterilisation of culture media.) This is the best method for sterilising *dry* glass-ware, such as test-tubes, Petri dishes, flasks, throat swabs, quill tubes, graduated and capillary pipettes, and certain instruments, such as forceps and scissors and *all-glass* syringes. Before sterilisation test-tubes or flasks should be plugged with cotton-wool stoppers which are covered with kraft paper (*vide* p. 90).

Although screw-capped bottles themselves will withstand the temperature of the hot-air oven, the rubber liners in the screw caps will not. Bottles already capped, therefore, cannot be sterilised by this method, but should be autoclaved (p. 84).

It should be noted that from certain brands of cotton-wool, volatile substances are given off during sterilisation; these condense on the tube and may interfere later with the growth of certain bacteria, *e.g.* pneumococcus.

Certain precautions have to be observed when sterilising glass-ware: (*a*) the glass should be perfectly dry; (*b*) the oven must be cold when the apparatus is inserted, then heated to the requisite temperature and kept at that temperature for the full time necessary for sterilisation; (*c*) the oven should be allowed to cool before the articles are removed, as sudden or uneven cooling is apt to cause cracking of the glass.

The advantage of this method is that all the articles are kept dry.

(3) *Flaming*, by passing the article through the Bunsen flame without allowing it to become red hot. This method is useful for sterilising scalpels, needles, the mouths of culture tubes, cotton-wool stoppers, glass slides and cover-slips. Needles and scalpels may also be sterilised by dipping them in methylated spirit and then burning off the spirit, the process being repeated several times.

STERILISATION BY MOIST HEAT

(1) *Boiling in a Water-Bath.*—A suitable form of steriliser is the fish-kettle type made of enamel-ware or tinned copper. It should have a removable tray provided with a raised edge to prevent cylindrical instruments, such as syringes, from falling off. Five minutes at 100° C. is sufficient to kill all non-sporing and many sporing organisms; the spores of certain species, however, may resist longer exposures, for one hour or more. This method of sterilisation has only a limited use in bacteriology, and is employed for tubes, instruments (forceps, scissors, etc.), syringes, pipettes, measuring cylinders, etc.. The addition of a small amount of sodium carbonate obviates rusting of steel instruments, but with stainless steel this is not necessary.

If the water supply is "hard," it is preferable to use distilled water, otherwise the instruments on removal become covered with a film of calcium salts.

The interior of a test-tube may be sterilised quickly for ordinary purposes by boiling water in it.

(2) *Steam at* 100° C.—This method is extensively used in bacteriology. A Koch or Arnold steam steriliser (sometimes called " steamer ") heated by steam, gas or electricity with an automatic regulator is employed, and is particularly useful for the sterilisation of culture media. In its simplest form this is a vertical metal cylinder with a removable conical lid (having a small opening at the top for the escaping steam) and containing water which is heated by a gas burner under the bottom of the cylinder. A perforated diaphragm situated above the water holds the articles to be sterilised. Its advantages are: (*a*) the apparatus need not be costly; (*b*) both the container and the medium are sterilised; (*c*) as the medium is in an atmosphere of steam, there is no loss from evaporation; (*d*) the apparatus requires little or no attention.

Sterilisation is effected in two ways :—

(*a*) One exposure for 1½ hours. This usually ensures complete sterilisation, and can be used for such media as broth or nutrient agar (*vide* p. 98). It cannot be used for nutrient gelatin (*vide* p. 103), as this medium, after prolonged heating, fails to solidify on cooling.

(*b*) Exposure at 100° C. for twenty minutes on each of three successive days. The usually accepted principle of this " intermittent " method of sterilisation is that one exposure is sufficient to kill the vegetative forms of bacteria; between the heatings, the spores, being in a favourable medium, become vegetative forms which are destroyed during the subsequent heating. This method, which is sometimes referred to as " Tyndallisation," is employed in sterilising media containing sugars (*vide* p. 119) which may be decomposed by higher temperatures or by prolonged heating. It is used also for the sterilisation of gelatin media.

It is advisable to cover the cotton-wool stoppers of tubes or flasks with two layers of parchment paper or kraft paper to avoid drenching.

(3) *Steam at High Pressure in the Autoclave.*—The principle on which the autoclave is used is that water boils when its vapour pressure is equal to the pressure of the surrounding atmosphere. If, therefore, the pressure be increased inside a closed vessel, the temperature at which the water boils will rise above 100° C., the exact temperature depending on the pressure employed. The usual form of laboratory autoclave consists of a vertical cylinder of gun-metal which is supported by a sheet-iron case. (The larger forms of autoclave have the cylinder arranged horizontally.) The lid is fastened by screw clamps, and is rendered air-tight by means of an asbestos washer. The cylinder contains water up to a certain level (*e.g.* 3½ in. for a vertical autoclave of 19 in. internal height) and this is heated by a Bunsen gas ring below the cylinder. The bottles, tubes, etc., to be sterilised are placed on a perforated diaphragm situated above the water level. The apparatus is furnished with a steam-tap and pressure-gauge, and also a safety-valve, which can be set to "blow off" at any desired pressure. The pressure originally employed was 15 lbs. per square inch above atmospheric pressure, making a total of 30 lbs. per square inch absolute pressure. Under this pressure water boils at 120° C., and fifteen minutes' exposure to this temperature (with few exceptions) kills all forms of organisms including spores. This pressure is still used for sterilising surgical dressings, empty glass containers, pathogenic cultures containing spores, etc.. When sterilising broth and agar, the high temperature resulting from 15 lbs. of steam pressure may have a detrimental effect on the nutritive properties of the medium. An exposure of thirty minutes at 5 lbs. pressure is preferable. Autoclaving must not be used for sugar media or gelatin, as the former are decomposed and the latter will not solidify on cooling.

Directions for using the Autoclave.—See that there is sufficient water in the cylinder. Insert material to be sterilised and light the gas. Place the lid in position, see that the tap is *open*, adjust the safety-valve to the required pressure[1] and screw down the lid. As the temperature inside the autoclave rises, air is forced out of the tap and eventually steam issues. *Make sure that all air has been expelled from the cylinder, as indicated by steam issuing freely*, and then close the tap. The pressure now rises until it reaches the desired level, when the safety-valve opens and the excess steam escapes. Allow the sterilisation to continue for the requisite time, fifteen or thirty minutes as the case may be, *from this point.* When sterilisation is complete, turn out the gas and allow the autoclave to cool until the pressure-gauge indicates that the inside is at atmospheric pressure. Now open the tap very slowly to allow air to pass into the autoclave, and remove the lid. If the pressure is suddenly released, the liquid media, being at a temperature above 100° C. and suddenly exposed to ordinary atmospheric pressure, will boil violently and be expelled from their containers with almost explosive force. In order to avoid drenching by the steam, cotton-wool stoppers should be covered with parchment paper or kraft paper.

Autoclaves heated by other means—*e.g.* steam or electricity—are operated in a similar manner.

Steam-heated Autoclaves.—In some laboratories autoclaves are heated by steam from the main steam supply. Such autoclaves are of the horizontal pattern, made of copper or gun-metal tinned inside, with a swing door fastened either with swing bolts and nuts, or by a "capstan head" which, on being turned, manipulates radial bolts. A convenient size is 30 in. diameter and 30 in. long (internal) fitted with two perforated shelves of stainless steel. The steam pipe entering the autoclave should have a baffle plate to distribute the steam and prevent it from impinging directly on the material to be autoclaved. A special reducing valve, which permits any pressure of steam from 0–15 lbs., is an advantage. It is important to have a thermometer, either of the ordinary mercury or recording type, fixed to the autoclave if steam is obtained direct from a steam main. When the steam is turned on the pressure-gauge rapidly registers the desired pressure, whereas the thermometer may show that the necessary temperature has not yet been reached. The duration of sterilising should be reckoned from the time when the proper temperature is attained.

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[1] In some varieties of autoclave the adjustment of the safety-valve has to be determined previously by trial.

Attention should be paid to the steam trap of the autoclave. If a bottle or tube containing agar breaks during sterilisation and the agar gains access to the trap and solidifies there, the autoclave may become half-filled with condensed water, which is unable to escape.

Autoclaving in "free steam."—A useful method when the autoclave is connected to a main steam supply is to sterilise by "free steam." The culture medium is placed in the autoclave, which is then tightly closed, and the steam supply turned on. When all the air has been expelled from the autoclave, steam issues freely from the escape-tap. Adjust the steam supply so that an adequate amount of steam is emitted from the autoclave, and allow this to continue during the period of sterilisation. Although the interior of the autoclave is open to the air the steam pressure inside is about 1–2 lbs. (above atmospheric pressure), which maintains the temperature just over 100° C. while the latent heat of the steam quickly warms up the contents of the autoclave, and maintains them at 100° C. or over during the time of sterilisation, which can be less than with the ordinary steam steriliser.

Alternatively, the contents of the autoclave can be thoroughly heated up by half-an-hour's exposure to "free steam," the escape-tap is then closed and the contents raised to 5 lbs. pressure for fifteen minutes. This is a very useful method for culture media such as broth and agar.

OTHER METHODS OF STERILISATION BY HEAT

The sterilisation of serum or body fluids containing coagulable protein can sometimes be effected by heating for one hour at 57° C. on several successive days. The principle is the same as in the case of intermittent sterilisation at 100° C. (*vide* p. 83). It may be necessary to repeat the heating eight times to ensure complete sterilisation. Care must be taken not to allow the temperature to rise above 59° C., as inspissation may occur. The exposure to 57° C. is best carried out in a water-bath, but a 57° C. oven may be used. This procedure is not always effective—*e.g.* if certain resistant types of sporing organisms are present.

Vaccines.—Vaccines should be sterilised in a special water-bath (" vaccine bath ") at a comparatively low temperature, one hour at 60° C. being *usually* sufficient. Higher temperatures may diminish the immunising power of the vaccine.

The *vaccine bath* consists of a copper container sometimes lagged with linoleum or other non-conducting material and is heated either by gas or electricity. The bath is fitted with a suitable removable rack for holding the tubes, while the lid has a hole into which a thermometer is placed. The bath is maintained at a constant temperature, usually 60° C., by means of a thermostatic control, which is either a "capsule", similar to that in an incubator, or a bimetallic device. When the temperature rises above the desired level, the thermostatic control diminishes the gas supply or cuts off the electricity. When the temperature falls the heating is resumed. By means of an adjusting screw the temperature can be regulated to ± 1° C. of that required. The bath should be inspected at weekly intervals and any loss of water, due to evaporation, etc., restored.

METHODS OF STERILISATION EMPLOYED FOR SPECIAL PURPOSES

STERILISATION BY CHEMICALS

(1) Volatile antiseptics, *e.g.* chloroform. — This method is sometimes used in the sterilisation and preservation of serum (for culture media), and the chloroform, which is added in the proportion of 0·25 per cent., can later be removed by heating at 57° C. If the serum is to be used for making a coagulated serum medium (*e.g.* Löffler's medium) the chloroform will be removed by the heating applied for coagulation. Chloroform is used also for preserving media in bulk.

(2) Antiseptics of the phenol group.—Liquor cresolis saponatus (lysol) and cresol are powerful antiseptics. Their chief use in a laboratory is for sterilising surgical instruments and discarded cultures, and for disinfecting the hands and killing cultures accidentally spilt by the worker. Lysol is generally used in a 3 per cent. solution. Phenol, 0·5 per cent., or *p*-chloro-*m*-cresol, 0·1 per cent., is used for preserving sera and vaccines (*vide* p. 287).

(3) Metallic salts or organic compounds of metals.—One of the most important in this group is perchloride of mercury (1 : 1000 solution). A bowl of this solution (conveniently prepared from tablets) may be kept on the laboratory bench for sterilising the hands after working with infected material or cultures.

"Merthiolate," a proprietary name for sodium-ethyl-mercuri-thiosalicylate, is used in a dilution of 1 in 10,000 for the preservation of antitoxic and other sera.

(4) Glycerol.—This is used as a 50 per cent. solution for the preservation of certain of the viruses, which retain their virulence in it for many months. In addition the glycerol kills off contaminating organisms, so that in time the virus-material contains no living bacteria of the ordinary type. This method is used in the preparation of vaccinia virus for vaccination against smallpox (*vide* p. 619). Glycerol is also used as a preservative in agglutinating and other sera (*vide* p. 261).

STERILISATION BY FILTRATION

This is effected by the use of filters of unglazed porcelain (Chamberland, Maassen type) or of diatomaceous earth (Berkefeld, Mandler clay filter), the pores of which are so small that ordinary bacteria are prevented from passing through.

Seitz filters, in which filtration is effected through asbestos "disks," are very useful and reliable. The actual filter consists of a flat disk of asbestos material of special composition, and is inserted into metal holders which ensure a tight joint being made. After use the asbestos disk is discarded and a new one employed for each filtration. The disks are inexpensive, and filters of various sizes may be obtained according to the amount of fluid to be handled. The large size of Seitz laboratory filter, with 14 cm. diameter disks, can be recommended for the sterilisation of large amounts of serum to be used in the preparation of media (*vide* p. 127). The K. type of disk is used for clarifying and the E.K. type for sterilising.

Asbestos disks, similar to, and as efficient as, Seitz filters, are now made in England. The G.S. type is used for sterilising and corresponds to the E.K., while the F.C.B. type is used for clarifying.[1]

Extremely fine filters made of collodion membranes are used in the study of the filterable viruses.

Further details are given on pp. 599, 645.

MAINTENANCE OF STERILITY

It is necessary that apparatus, after sterilisation, should be kept sterile.

Test-Tubes and Flasks.—The interiors of test-tubes, flasks, bottles, etc., must be carefully protected from bacterial contamination due to access of air, dust, etc., before and after the addition of medium and during the subsequent cultivation of organisms. This has usually been done by means of cotton-wool stoppers. These should be $1\frac{1}{4}$–$1\frac{1}{2}$ in. long, $\frac{3}{4}$–1 in. being inserted into the mouth of the tube, etc. and the remainder projecting. They should fit firmly, but not so tightly as to render their removal difficult.

Stoppering of Tubes.—Long-fibre cotton-wool is essential, and must be free from short broken fibres and dust. Non-absorbent cotton is preferable, because, after steaming, plugs tend to remain moist, and if the medium is to be kept for any length of time and absorbent wool is used, moulds will grow through the stopper and contaminate the medium. A sufficient amount of cotton-wool (*vide supra*) should be forced into the tube with a rod or pair of forceps, but should not be twisted in, as creases are formed along the sides of the glass and create channels for contaminating organisms.

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[1] Supplied by A. Gallenkamp & Co. Ltd., London.

Instead of the ordinary roll of cotton-wool being used, it is recommended that the non-absorbent wool be obtained in the form of a long thin ribbon known as " rope wool " or " neck wool " of the type used by hairdressers. It is kept in a tin container with a hole in the lid, and the appropriate amount of wool for the stopper is easily obtained without waste.

When tubes or flasks have to be stored for some time the stoppers or tops of the crates or boxes should be covered with sterile kraft paper, kept in place by means of fine string or a rubber band. Sterile rubber stoppers may, in some cases, be used instead of cotton-wool, particularly where the contents of the flask or tube have to be kept a considerable time, as in the case of immune sera ; this also applies to vessels which have to be transported by post or by messenger.

Screw-capped Bottles.—Flasks for storing culture media have now been replaced by screw-capped bottles of 2, 4, 6, 10 and 20 oz. capacity, while the smaller bottles of $\frac{1}{4}$, $\frac{1}{2}$ and 1 oz. capacity are employed instead of test-tubes. Their use is referred to later.

Petri Dishes.—Each individual dish should be wrapped in kraft paper before sterilisation, and kept in the paper until used. For a 4 in. dish the size of paper should be 12 in. square. The dishes may also be sterilised (unwrapped) and kept in cylindrical tinned-copper boxes.

Pipettes.—1 c.c. and 10 c.c. graduated pipettes should be wrapped in a long strip of kraft paper, which is wound round them in a spiral manner before sterilising in the hot-air oven. Bulb pipettes (10 c.c., 50 c.c., etc.) are also covered with kraft paper. Under these conditions pipettes remain sterile in their wrappers for considerable periods of time.

Capillary pipettes are sterilised in large test-tubes, 15 in. × $2\frac{1}{2}$ in., having a cotton-wool stopper, or in tinned-copper boxes. The former method is preferable. Alternatively, 8 in. lengths of 5 mm. glass tubing are plugged with cotton-wool at both ends, wrapped in batches of a dozen in kraft paper, sterilised and stored. When capillary pipettes are required, the middle of the tubing is heated in a Bunsen or blowpipe and pulled out, the ends of the two pipettes being sealed in the making.

Ampoules are sterilised in the hot-air oven with the necks sealed and are kept in metal boxes. If unsealed ampoules are used, they should be plugged with cotton-wool before sterilisation.

PREPARATION OF CULTURE MEDIA

The majority of the organisms to be studied are pathogenic, and in order to obtain suitable growths the artificial culture media should approximate to the composition and reaction of the tissues and body fluids in which these organisms grow.

FOOD SUPPLY AND ENERGY REQUIREMENTS OF BACTERIA

Most bacteria derive their energy by oxidative decomposition of organic food material such as proteins and carbohydrates. At the same time simple organic substances—*e.g.* amino-acids and other products of protein disintegration—are utilised in the synthetic metabolism of these organisms.

The food requirements of bacteria vary with the natural environment and the particular rôle they have in nature. Some organisms are able to grow under a wide range of conditions, whereas other more highly parasitic bacteria, such as the gonococcus, are restricted in their requirements, with regard not only to food but also to temperature and other factors. It is usually impossible to reproduce exactly the natural conditions under which pathogenic micro-organisms flourish. On the other hand it must be realised that a considerable degree of adaptability exists among them, and for the great majority of pathogenic bacteria suitable artificial media have been found.

Bacteria require nitrogenous food material, and in the case of the pathogenic organisms this generally consists of protein derivatives. In the usual culture media nitrogen is conveniently supplied in the form of "peptone" which is a commercial product obtained by peptic digestion. Peptone is a crude product and consists of a mixture of proteoses, peptones, polypeptides and amino-acids. This simple digested form of protein is soluble and does not coagulate on heating. It can therefore be incorporated in media which later have to undergo sterilisation by heat. Moreover, it is well adapted to the synthetic metabolism of bacteria, especially if it contains a sufficiency of amino-acids. Alternatively, instead of adding the digested protein in the form of commercial peptone, native proteins are split up during the preparation of the medium by the action of pepsin or trypsin to form simpler uncoagulable nitrogenous compounds. This is the principle of the so-called "digest media," of which Hartley's broth (*vide* p. 101) is an example. Other forms in which nitrogenous material is incorporated in culture media are animal serum—*e.g.* serum-agar and Löffler's medium—and egg protein as exemplified by Dorset's egg medium for the tubercle bacillus, but bacteria do not directly utilise complete proteins.

Carbon as a constituent of bacterial protoplasm is derived mainly by the breaking up of carbohydrates. This process is essential in the case of many organisms. Carbohydrates are supplied generally in the form of sugars (*vide* p. 119), and if fermented they are usually valuable in promoting growth—e.g. *B. diphtheriae* grows much better if a small quantity of glucose is added.

Various "growth factors" are important in the cultivation of many pathogenic bacteria, and these can be supplied in *fresh* body fluids, such as blood, serum and ascitic fluid, which at the same time may yield other nutritive substances. It must be recognised that these growth factors are often susceptible to heat and adsorption, and that excessive heating and filtration of media must be avoided if they are to be retained.

Mineral salts are essential to growth, particularly the chlorides, phosphates and sulphates among the acid radicles, and calcium, potassium and sodium among the bases.

The value of meat extract incorporated in culture media is recognised although its precise properties have not yet been fully determined. In composition it is practically devoid of protein, and consists of mineral salts, a small amount of sugar and some nitrogenous muscle extractives. It probably supplies certain amino-acids and growth factors.

There are certain pathogens with particular requirements which have to be fulfilled before growth takes place. Some highly parasitic organisms may require the presence of serum—*e.g.* the gonococcus. The haemophilic bacteria—e.g. *B. influenzae*—do not grow on ordinary media, but multiply if blood is present; two growth-promoting factors present in blood, designated "X" and "V", appear to be essential. The "X" factor is thermostable and resists autoclaving at 120° C. It consists of haematin, and the suggestion has been made that it is required for the synthesis of catalase which is essential for the aerobic growth of the organism (*vide* p. 496). The "V" factor is more easily destroyed by heat, and at one time was considered to be a vitamin but has now been shown to be an enzyme, coenzyme I (cozymase). Egg media are especially suitable for the growth of the tubercle bacillus. The bacillus of Johne's disease can be grown if an extract of other acid-fast bacilli is present, and it would appear that some substance is synthesised by these organisms which is necessary for the growth of the former. The filterable viruses are unable to multiply on the usual artificial media, but many of them have been cultivated in association with embryonic cells as in tissue cultures or with surviving tissue cells.

Certain atmospheric conditions also are essential for growth of various bacteria; these are dealt with on pp. 18, 172.

Bacterial Nutrition.—In recent years attention has been directed towards the essential requirements of bacterial nutrition, and the mechanism by which growth is obtained.

The classes of bacterial nutrients, *e.g.* nitrogenous, carbonaceous, mineral, etc., have already been enumerated above, but it is the enzymic content of the bacteria that determines which substances can be utilised for growth. In this country the main work on the subject has been carried out by Fildes and his colleagues. By using simple solutions of amino-acids as a basis, it can be shown that certain well-defined chemical substances are necessary for the growth of particular bacteria. Thus, it was found that pantothenic acid was necessary for the growth of yeast, and later the first two chemically known substances essential for bacterial growth were described, namely riboflavin for certain lactic acid bacteria and uracil for staphylococci grown anaerobically. More recently numerous other essential growth factors have been defined, *e.g.* glutamine for streptococci, pimelic acid and nicotinic acid for *B. diphtheriae*, etc..

It has been shown that free-living, non-parasitic bacteria are capable of synthesising for themselves the various metabolites necessary for their growth. They have the requisite enzymes to produce these substances by catalysis from more simple compounds. Presumably, as an organism becomes more parasitic, so it obtains increasingly its essential metabolites from the host, and loses the function of elaborating them for itself. In the most highly parasitic organisms there is considerable lack of enzymes, and in consequence such species are more difficult to grow on artificial media and in fact cannot be grown satisfactorily unless the essential metabolites are added.

Sometimes, however, an organism while unable to synthesise a growth factor from simple compounds, is able to utilise to some extent substances closely related to this factor. Thus, a streptococcus which requires glutamine can utilise glutamate. It has been shown also that bacteria may be "trained" to grow without the essential growth factor being added, by offering in its place another material from which it is thought the organisms might be able to synthesise the necessary growth substance. Once this has been achieved, successive subcultures maintain the synthesising property. This is what happens when a highly parasitic organism, at first difficult to cultivate on an artificial culture medium, becomes adapted to and flourishes on the medium and later grows abundantly on even simpler media.

It is thus seen that with the development of the parasitic habit, organisms have lost the capacity to synthesise their essential metabolites. As they have become more and more parasitic they have lost not only their enzymic activity but also their substance, as illustrated by the viruses which are exceedingly small and are unable to synthesise any growth metabolites, obtaining the essential enzyme-systems only from the living cell. This explains why the viruses cannot be cultivated on artificial media, or on cell extracts, but only in the living cell (*vide* p. 595).

BOTTLED CULTURE MEDIA

Hitherto culture media have been stored in bulk in flasks with cotton-wool or rubber stoppers, or in some form of "milk" or "whisky" bottle. In recent years the use of screw-capped bottles which enables the medium to be stored[1] has become widespread. This consists essentially in placing the medium in a container, which is then hermetically sealed and thereafter sterilised. The result is that, the container being completely closed, the contents remain sterile indefinitely, in exactly the same way as canned foods. There is no necessity to store in a cold room, while transport is much more easily accomplished. The principle is applied to media not only in bulk, but also in smaller quantities, and small screw-capped bottles can be substituted for test-tubes for bacteriological purposes. It should be noted that though the use of screw-capped bottles was introduced to facilitate the distribution, storage and transport of culture media in a large laboratory organisation, it can equally well be applied in smaller laboratories, and where comparatively small quantities of culture media are made and used. The methods of using these bottles are particularly recommended, as considerable saving in material and labour will result.

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[1] See J. E. McCartney, "Screw-capped Bottles in the Preparation and Storage of Culture Media," *Lancet*, 1933, ii, 433.

The original methods of employing flasks and test-tubes as containers of culture media are given throughout this chapter, but the practical application of the above-mentioned system is also described.

For culture media in bulk, screw-capped plain white bottles are employed. They are supplied in various sizes, ranging from 2 oz. to 20 oz., but the sizes most commonly used are 2 oz., 4 oz., 10 oz. and 20 oz., in which are placed 50, 100, 250 and 500 c.c. of media respectively. As a substitute for test-tubes small round screw-capped bottles of $\frac{1}{4}$, $\frac{1}{2}$ and 1 oz. capacity are used. The bottles are made of clear white flint glass and the neck has an external screw thread. The screw caps are made of aluminium (the smaller sizes are sprayed with white cellulose paint) and the washer is made of red rubber.

Types of Screw-capped Bottles.—Screw-capped bottles are now extensively used in laboratory work and the following range, which covers practically all needs, indicates the most useful types.

Details of the caps are given to show how comparatively few varieties are needed for a complete series. It should be noted that six of the smaller bottles (which are the most used) need only two sizes of caps, which is economical in supplying and maintaining stocks. Bottles may be obtained with the caps perforated (for blood cultures, p. 185).

Bottle.	*Capacity. in c.c.*	*Cap.*	*Washer.*
1 gallon, narrow mouth	4600	Special, to fit	"Compo" cork and "resistol" liner.
80 oz. Winchester series	2400	Charlton senior aluminium	3 mm. red rubber.
[1] 40 oz. ,, ,,	1190	Ditto	,, ,,
[1] 20 oz. ,, ,,	600	Ditto	,, ,,
10 oz. ,, ,,	290	Charlton junior aluminium	2 mm. red rubber.
6 oz. round "Limes"	170	M.3 sprayed white	,, ,,
4 oz. ,, ,,	115	M.3 ,,	,, ,,
2 oz. medical flat .	60	M.2 ,,	,, ,,
[2] 1 oz. round (H 53) .	28	M.3 ,,	,, ,,
½ oz. ,, .	15	M.2 ,,	,, ,,
¼ oz. ,, (bijou) .	6	M.2 ,,	,, ,,
1 oz. universal container	28	Special, to fit; aluminium lacquered	,, ,,

As "medical flat" bottles with the cap on may crack when sterilised in the autoclave, their use in laboratory work has been discontinued except in the smallest sizes. The round bottles stand autoclaving repeatedly with scarcely a casualty.

These bottles are made by the United Glass Bottle Manufacturers, Ltd., 8 Leicester Street, London, W.C.2. With the exception of the first two, they are supplied cleaned and washed by a special process which removes the surface alkali. The caps are already fitted, the rubber washers having previously been well boiled before insertion. No further treatment is necessary before they are used, and culture media can be added to them after which they are capped and the contents sterilised in the appropriate manner. The introduction of this new process of cleaning and washing by the makers saves all the tedious work of preparing new glassware in the laboratory, which is time-consuming and expensive. It is of especial value where large quantities of culture media are produced. Normally the cleaned bottles are supplied in cardboard cartons which not only keep the bottles clean and facilitate storage before use, but are very useful for storing and despatching the culture medium after it has been made.

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[1] These bottles are also used for intravenous infusion solutions, *e.g.* saline, glucose-saline, etc., and as blood transfusion bottles.

[2] See Fig. on p. 161.

These bottles can be autoclaved with the caps tightly screwed on, either empty or containing media, with little risk of breakage. Care must be taken that the bottles are placed in the steriliser loosely, and not packed tightly in a wire crate or other container, otherwise breakages will inevitably occur. They must not be sterilised in the hot-air oven with the caps on, as the temperature will injure the rubber washer.

In addition to the bottles the following screw-capped containers are useful in laboratory work :—

2 oz. " pomade pot." See p. 318.
6 oz. " honey pot." See p. 319.
½ lb. jar } same size of cap. } See p. 321.
1 lb. jar }
½ gallon wide-mouth jar } For the preparation of culture media.
1 gallon ,, ,, ,, }

BROTH (or BOUILLON), NUTRIENT GELATIN AND AGAR

The basis for the media ordinarily employed in the study of the common pathogenic bacteria is nutrient broth. Agar or gelatin is added merely to solidify it.

It should be noted here that the various " digest " media to be described are of great value for obtaining very luxuriant growths of organisms, but the cultures may die out rapidly. For maintaining stock cultures the use of media prepared from ordinary meat extract is advisable.

BROTH

The first stage in the preparation is the making of a watery extract of meat. The type of meat used is an important factor in the quality of the broth obtained. Freshly killed (not frozen) lean beef or ox heart should be used. Horse-flesh is cheaper, but is usually not so fresh, and, in addition, contains a higher percentage of fermentable sugar, which may make the broth unsuitable for many purposes, such as the preparation of toxins. The meat is carefully freed from fat, minced as finely as possible, and added to tap water in the proportion of 500 grams to 1 litre. After extraction for twenty-four hours at a low temperature—*e.g.* in the refrigerator—the mixture is strained through muslin to keep back the small particles of meat, and the meat residue expressed. The fluid is bright red in colour and there is often a thin layer of fat on the surface, which may be removed by skimming with a piece of filter paper. It is boiled for fifteen minutes, or steamed in a steam steriliser for two hours, when it becomes brown in colour and turbid on account of the alteration of the haemoglobin and the small particles of coagulated protein. It is now filtered and the clear fluid is made up to the original volume by the addition of distilled water. The unheated material contains soluble proteins along with other extractives. As a result of the heating the meat proteins are coagulated, and are removed by filtration. The finished extract should be clear and light yellow in colour, but is not suitable as a culture medium owing to the lack of nitrogenous material. Digested and uncoagulable protein in the form of commercial peptone[1] is added in the proportion of 1–2 per cent., and the salt content is increased by the addition of sodium chloride (0·5 per cent.). These are dissolved by heat and the extract is again filtered. Owing to the sarcolactic acid present in the meat, the reaction of the extract is acid, and this reaction is unsuitable for the growth of most organisms.

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[1] For ordinary purposes "commercial" peptone is satisfactory, but for special purposes sugar and indole must be absent.

When the reaction has been adjusted to the optimum by suitable means (*vide* p. 113), the medium is sterilised in the autoclave or steam steriliser, and the resulting preparation is designated Infusion Broth or Bouillon.

In the preparation of culture media it should be noted that very small quantities of copper salts are inimical to the growth of many organisms. Copper utensils should be avoided, but heavily tinned copper articles, *e.g.* funnels, containers, are safe to use. Should the tinning show signs of wear the article must be re-tinned. Commercial brands of peptone may contain copper salts derived from the vessels used in their manufacture, and it is essential that any peptone used should be copper-free.

It has been shown by H. D. Wright that broth may be unsuitable for the cultivation of the more delicate organisms if the peptone is incompletely reduced. When the peptone is added to the meat and water and the whole boiled together, the resultant broth gives good results, particularly with the pneumococcus. The broth, however, is not so suitable for the cultivation of many anaerobes such as the tetanus bacillus. *Wright's method* is as follows: To 1 litre of distilled water add 10 grams of peptone, 5 grams of sodium chloride, and 500 grams of meat, preferably veal, finely minced after removal of excess of fat. Mix well and heat for 20 minutes at 68° C., stirring at intervals. Shake well and steam in the steam steriliser for 30 minutes, filter through paper and adjust the reaction to pH 7·8–8·0 (*vide* p. 113). Again steam for 30 minutes and filter through paper. Check the reaction of the filtrate (pH 7·6–7·8) and add 1·5 grams of glucose per litre. Distribute as required and sterilise by autoclaving for 10 minutes at 10 lbs. pressure. This broth can be used for ordinary purposes, when the glucose may be omitted.

Lab.-Lemco.—A meat extract known as "Lab.-Lemco" may be used as a substitute for the extract of fresh meat in the proportion of 10 grams of Lab.-Lemco to a litre of water. The addition of salt (0·5 per cent.) and peptone (1 per cent.) converts it into ordinary broth. The reaction must be standardised as in the case of the ordinary infusion broth.

Infusion broth and nutrient agar made from it are identified by means of a YELLOW bead in the container (p. 157).

DIGEST MEDIA

In the ordinary media, digested protein is added in the form of peptone, but in the "digest" media the meat is digested by the action of trypsin, and the products of this digestion constitute the basis of the medium. The simpler uncoagulable nitrogenous compounds necessary for the growth of organisms are therefore not added as such, but are formed during the preparation of the medium.

A useful form of "digest" medium for general use, which is also particularly suitable for the production of diphtheria toxin, is :

HARTLEY'S BROTH

Ox heart or lean beef (free from fat and minced)	1500 grams
Tap water	2500 c.c.

Mix together and heat in the steam steriliser until a temperature of 80° C. is reached. Then add :

Sodium carbonate (anhydrous) 0·8 per cent. solution (cold)	2500 c.c.

Cool to 45° C. and add :

Pancreatic extract[1]	50 c.c.
Chloroform	50 c.c.

The mixture is then incubated at 37° C. for six hours, or 45° C. for 3 hours, the liquid being frequently stirred. After digestion is completed, add 40 c.c. of pure strong hydrochloric acid, steam for half-an-hour, and then filter. Adjust the reaction

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[1] COLE AND ONSLOW'S PANCREATIC EXTRACT

Fresh pig pancreas (fat-free and minced)	500 grams
Distilled water	1500 c.c.
Absolute alcohol	500 c.c.

Industrial methylated spirit may be used instead of absolute alcohol (*vide* note on p. 193).

Shake the mixture thoroughly in a large stoppered bottle and allow to stand for three days at room temperature, the shaking being repeated occasionally. Strain through muslin, and filter through Chardin paper. Measure the filtrate and add pure strong hydrochloric acid in the proportion of 0·1 per cent. This causes a cloudy precipitate which settles in a few days and can be filtered off.

Filtration of the extract is not essential and it can be kept exactly as mixed, with the addition of hydrochloric acid.

This extract keeps for several months in stoppered bottles. If used at once the hydrochloric acid need not be added as its action is to retard the slow deterioration of the trypsin.

with normal solution of caustic soda so as to be neutral to phenolphthalein (pH 8·4) and steam for one hour to precipitate phosphates. Filter while hot and allow to cool. Adjust the reaction to pH 7·6 and distribute in 250 c.c. and 500 c.c. amounts in 10 and 20 oz. screw-capped "Winchester" bottles, or in smaller amounts, *e.g.* in blood-culture bottles (p. 184) or in 1 oz. and ¼ oz. bottles according to requirements. The caps are tightly screwed on and the bottles sterilised.

If the broth is to be stored in bulk, the precipitate of phosphates is not removed after steaming. When the broth is cool, distribute into one-gallon screw-capped bottles. Add 0·25 per cent. of chloroform and shake vigorously. Shake the bottles frequently during the next 2 or 3 days and store in a cool dark place.

Digest broth and the nutrient agar made from it are identified by a BLACK bead in the container (p. 157).

HORSE FLESH DIGEST MEDIUM

This medium is specially suitable for cultivating haemolytic streptococci when an abundant growth is required.

Mix 2 lbs. minced horse flesh with 1500 c.c. cold water and raise temperature to 80° C.; add 2000 c.c. cold water and 12 grams sodium carbonate (anhydrous). Adjust pH to 8. Add 0·5 per cent. pancreatin and keep at 56° C. for six hours; then add 20 c.c. pure hydrochloric acid, boil for half-an-hour to arrest digestion, and filter. Incorporate in the digest 1 per cent. de Fresne's peptone (or other high quality peptone) and adjust to pH 8; add 0·125 per cent. calcium chloride; steam and filter when cold. Add 0·2 per cent. sodium bicarbonate and filter through a Seitz filter; store in bottles; incubate at 37° C. to test for sterility.

Broth being a fluid medium has certain disadvantages:

(1) growths do not exhibit specially characteristic appearances in this medium, and therefore it is of little use in identifying species;
(2) organisms cannot be separated from mixtures by growth in this medium (*vide* p. 167).

It can, however, be rendered solid by the addition of gelatin, 10–15 per cent., or agar-agar, 2 per cent.

NUTRIENT GELATIN

This medium, first introduced by Koch, is made by dissolving the best sheet-gelatin, 10–15 per cent. by weight, in broth. Solution is effected by heating in a steam steriliser. The product is acid and is made alkaline to phenolphthalein. The mixture is filtered and cleared with white of egg (see Agar). The finished product is adjusted to pH 7·6 and sterilised in the steam steriliser—twenty minutes each day on three successive days. Prolonged exposure to 100° C. or autoclaving destroys its property of solidifying when cooled, thus rendering it useless for bacteriological purposes.

The resulting medium is perfectly transparent when solid, and should be of firm consistence, yet not so stiff that it is split by the wire when inoculated (*vide* p. 160).

The proportion of gelatin used varies with the time of year, and in very hot summer weather 15 per cent. may be necessary. A suitable consistence may be obtained by adding 10 per cent. in the winter and 12 per cent. in the summer. Gelatin at this strength melts at about 24° C., and is therefore fluid at incubator temperature. Organisms, however, can be grown at 37° C. and liquefaction then tested by placing the culture in cold water.

Gelatin is a protein, and therefore is digested and liquefied by the proteolytic ferments of many bacteria. This property of liquefying gelatin is used as a means of differentiating certain organisms. As gelatin is not frequently used in routine work it should be kept in small screw-capped bottles (*vide* p. 161). When the medium is made it is distributed into the bottles, the caps are tightly screwed on, and the whole batch is sterilised. Under these circumstances the medium keeps indefinitely. After the gelatin has been inoculated (*vide* p. 161) the cap is again screwed on, which prevents evaporation during the growth of the organism.

NUTRIENT AGAR

Agar-agar, or " Agar " for short, comes into the market in the form of dried strands prepared from a seaweed found in the Japanese and Chinese seas. It is also obtained powdered, which is preferable.

Agar powder or fibre, as bought, contains many solid impurities, and when nutrient agar is made it must be filtered to produce a clear and transparent medium. Owing to the large amount of minute colloidal particles present, filtration through filter paper is slow, and in consequence before filtration the nutrient agar is " cleared " with egg albumin.

The proportion of agar generally used to solidify broth is 2 per cent. of the powder, and 2·5 per cent. of the fibre. After the agar has been added, the broth is placed in the steam steriliser for one hour to effect solution. The mixture is then cooled to 55° C., and the switched whites of two eggs, or 10 grams of egg albumin dissolved in 50 c.c. water, are added per litre.[1] The medium is now steamed for two hours, or preferably autoclaved for one hour at a pressure of 5 lbs. per square inch. The agar solution is then filtered through Chardin filter paper in the steamer, distributed into bottles or tubes, and finally sterilised either by autoclaving at 5 lbs. pressure for half-an-hour, or by steaming for half-an-hour each day on three successive days.

When dealing with large quantities of medium, it will be found quicker and easier to filter nutrient agar through sand and paper pulp[2] instead of through filter paper. For amounts up to five litres we can recommend the following method. Into a 10 in. glass funnel place about a dozen round clean pebbles to form a support for the sand and paper pulp. The paper pulp is thoroughly shredded and soaked in warm water. Take a large sheet of muslin 24 in. square, and place it over the funnel with the ends hanging over the side. Place moist paper pulp on top of the muslin until the layer is two inches deep, and pack it firmly (with the bottom of a 100 c.c. cylindrical measure), especially round the edge. Turn the ends of the muslin over the surface of the paper pulp, so as to form a sort of bag. Now superimpose on this a one-inch layer consisting of equal parts of moist paper pulp and well-washed finely crushed flint. The funnel and its contents are placed in the steamer or autoclave at the same time as the broth and agar powder when the latter is to be dissolved. It is important to note that no egg or egg albumin is required. The funnel is taken while hot from the steriliser, connected to the water-pump or other suction apparatus (*vide* p. 638), and melted agar poured into it. Gentle suction is applied and the water in the pulp and sand first runs from the lower end of the funnel and after

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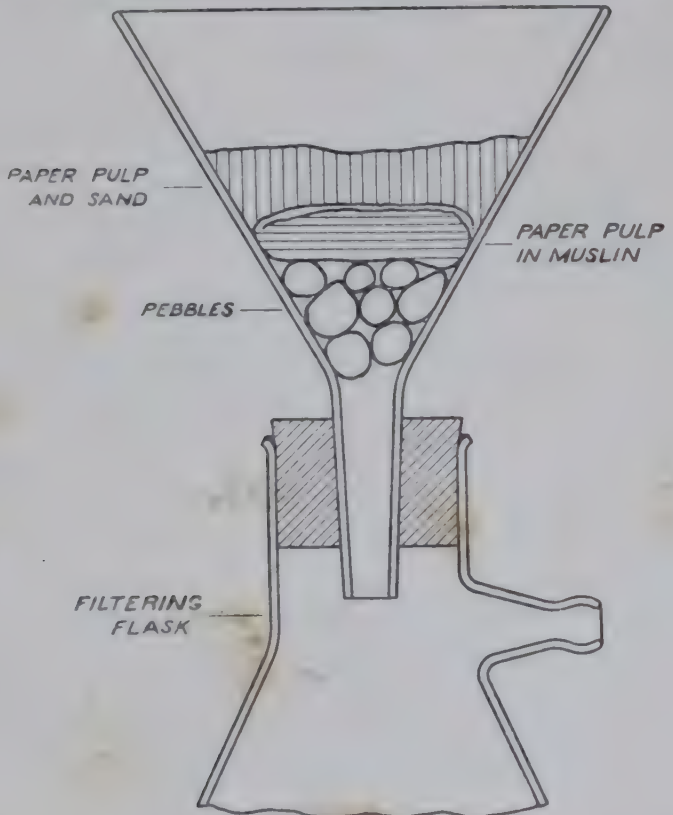
[1] As an alternative to eggs, 40 c.c. of ox serum per litre of medium may be used.

[2] A suitable paper pulp is " White Heather " brand, also T. B. Ford's filter pulp. Both these are sold in slabs.



a few seconds agar begins to appear. Suction is stopped, the water and dilute agar discarded, and the filtration now proceeded with. As the filtration continues the amount of suction should be slightly increased. The agar passes through very rapidly, and with the apparatus figured five litres can be filtered in thirty to forty minutes. It is not advisable to filter any greater quantity without changing the paper pulp and sand. For larger quantities of medium, a one- or two-gallon cylindrical stone jar with a false bottom or a specially made tinned copper container is used, and amounts of agar up to fifty litres an hour can be dealt with.

This agar-broth medium is known as Nutrient Agar or " Ordinary Agar."

When fluid, it is perfectly clear, but when solidified is faintly opalescent. Its advantage is that it is solid at 37° C. which is the optimum temperature for most pathogenic organisms. *The medium must be heated to* 98° C. *to melt it, but when melted it may be cooled down to* 42° C. *before solidifying.* This property is utilised in the preparation of serum-agar and blood-agar (*vide* p. 129). The agar may thus be cooled below the coagulating point of the serum proteins—60° C.—and yet remain fluid so that the serum or blood is present unaltered in the medium.

Agar-agar differs from gelatin inasmuch as it is a carbohydrate and is not generally liquefied by bacteria.

Nutrient agar made from infusion broth is distinguished by a YELLOW bead in the container, whereas nutrient agar made from digest broth (Hartley's broth) is identified by a BLACK bead (p. 157).

Both broth and agar media may be enriched or modified by the addition of various substances.

GLUCOSE BROTH.—Broth *plus* ½ or 1 per cent. of glucose. Since glucose acts as a reducing agent, this medium may be used for the growth of anaerobes.

GLYCEROL BROTH.—Broth to which glycerol is added in the proportion of 5–8 per cent. This medium is sometimes used for the growth of the tubercle bacillus.

Similarly, GLUCOSE AGAR is made by the addition of ½-1 per cent. of glucose, and GLYCEROL AGAR by adding 5–8 per cent. of glycerol to ordinary agar. The former is used chiefly for deep stab cultures of anaerobes (*vide* p. 173).

GLADSTONE AND FILDES' MEDIUM (Casein-Yeast Agar)[1]

This medium dispenses with meat extract and peptone. Its cost of production is small and most organisms generally met with will grow on it. The basal stock solutions keep indefinitely, so that successive batches are simple to make and are uniform in quality.

The necessary amino-nitrogen content of the medium is supplied by an acid hydrolysate of casein. Since acid hydrolysis partially destroys certain amino-acids, *e.g.* tryptophane, the hydrolysate is supplemented with a small quantity of tryptic digest of casein. Watery extract of yeast and glutamine are added to provide most of the essential growth factors. The medium is buffered with sodium glycerophosphate, which may also act as a source of energy. Sodium lactate is added as a further source of energy. Other additions, *e.g.* blood and serum, may also be made, depending on the purpose for which the medium is required.

STOCK SOLUTIONS AND SUBSTANCES

(A) *Preparation of stock hydrolysed casein.*—To 200 grams of commercial casein in a litre conical beaker, add a mixture of 170 c.c. concentrated HCl with 110 c.c. distilled water. Stir quickly with a glass rod to obtain a uniform suspension before the casein swells and becomes solid. Autoclave at 120° C. for ¾ hour. Cool and add 40 per cent. NaOH till neutral (about 180 c.c.); cool again and filter through pulp[2] on a Buchner funnel. Dilute to 1 litre, place in a Winchester quart bottle and add 1 per cent. chloroform. Shake vigorously immediately and at intervals to emulsify the chloroform. Store in the dark. One litre of this solution makes about 30 litres of complete medium.

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[1] For full details, including modifications for special purposes, see Gladstone, G. P., and Fildes, P., *Brit. J. Exp. Path.*, 1940, **21**, 161, from which this account is taken.

[2] The pulp may be T. B. Ford's 4 per cent. asbestos-cotton filter pulp, sold in slabs. For use, 5 grams are thoroughly suspended in 500 c.c. of distilled water. The suspension is placed on a 5½ in. Buchner funnel, already protected with a No. 1 Whatman filter paper, and allowed to drain by gravity. It is then sucked dry and covered with another No. 1 filter paper (see also footnote, p. 104).

(B) *Stock tryptic digest of casein.*—Place 200 grams of casein ("light white") and 20 grams sodium carbonate crystals in a Winchester quart bottle. Add 2 litres of warm (37° C.) tap water in two lots of 1 litre each, shaking well after each addition. Add 100 c.c. of a suspension of minced pancreas[1] and 10 c.c. chloroform, shake well and test pH which should be 7·4–7·5 (p. 114). Incubate for sixteen days at 37° C., shaking each day. Samples should be taken daily to check the pH, which should be adjusted if necessary.

After removal from the incubator, allow to stand for two days and filter off the tyrosine crystals, which settle as a white deposit. To 2 litres of the filtrate add 15 c.c. of concentrated HCl in 150 c.c. of water and steam for one hour.

Cool, filter and adjust the reaction to pH 7·4 (6–7 c.c. of 40 per cent. NaOH are required). Place in a Winchester quart bottle, add 1 per cent. chloroform, shake thoroughly at once and at intervals to keep the chloroform emulsified, and store in the dark.

(C) *Stock extract of yeast.*—Crumble 250 grams pressed brewer's yeast into 1 litre of distilled water, boiling over a naked flame. Stir continuously until frothing ceases (about five minutes). Filter on a Buchner filter through pulp. Store in Winchester quart bottles in the dark, after adding 1 per cent. chloroform and shaking vigorously.

For mass production, each boiling may be pooled and left in the cold overnight. The supernatant fluid is then syphoned off, filtered through pulp and bottled as above. The deposit may be discarded.

(D) *Sodium glycerophosphate.*—This is kept as the solid salt of commercial grade.

(E) *Glutamine.*[2]—A 0·05 per cent. solution in distilled water is sterilised by filtration and kept in the ice-chest. Glutamine solutions are not stable indefinitely and small quantities only should be made up. The stock of glutamine crystals should be preserved in a desiccator in the ice-chest. Glutamine is used in a final concentration of 0·00025 per cent.; 1 gram, therefore, will be sufficient for 400 litres of medium.

(F) *Sodium lactate.*—Commercial sodium lactate may be bought as a 50 per cent. syrupy solution.

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[1] Cole and Onslow's pancreatic extract (footnote, p. 101).

[2] Obtainable from the British Drug Houses, Ltd.

PREPARATION OF THE BASAL CASEIN-CASEIN-YEAST MEDIUM (CCY)

Solid medium.—Mix 15 grams powdered agar in 850 c.c. distilled water, adding the water gradually to make a smooth paste and finally a uniform suspension. Place in the autoclave, already hot, and allow to steam for five minutes, then run up to 15 lbs. and turn off heat. Open in half-an-hour.

Take—

Casein (A)	35 c.c.
Casein (B)	15 ,,
Yeast (C)	100 ,,
Sodium glycerophosphate (D) . .	10 grams
Sodium lactate (F)	10 c.c.

Shake to dissolve the glycerophosphate, heat the mixture to 100° C. and mix with the hot (100° C.) agar solution. Add 3·6 c.c. of 40 per cent. NaOH (to increase the pH to about 9·3),[1] place in the steamer and test the temperature, which should not be less than 90°–95° C. At this point a precipitate forms and is allowed to deposit. Filter at once as follows: prepare a Buchner funnel with pulp as already described (footnote, p. 104); fit it to a filter flask attached to a water pump and stand in the steamer until the funnel is very hot. Turn on the pump and pour the hot agar on to the upper filter paper. To avoid prolonged heating at a high pH, filtration should be as rapid as possible. For this purpose the bulk of the precipitate, which soon settles to the bottom, should not be shaken up. The supernatant fluid passes through the filter rapidly (about five to ten minutes) and can be removed from the steamer. The small amount of residue may then be passed through the filter or may be discarded. The filtered agar must not be allowed to stand at this high pH; therefore, without delay, add 2·05 c.c. concentrated HCl (sp. gr. 1·142, 32 per cent.) per litre. If more than 1 litre of medium is being made, the NaOH should only be added to 1 litre at a time, this is filtered and the pH readjusted, before another litre is treated in the same way. Meanwhile the rest of the medium before the addition of NaOH is kept hot in the steamer. Not more than 1 litre should be passed through each filter. Finally pool the filtered agar and check the pH, which should be 7·6, cresol red being used as the indicator.

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[1] To remove phosphates present in the commercial glycerophosphate.

Add : Glutamine solution (E) [1] . . 5 c.c. per litre.

Distribute in bottles and autoclave.

Liquid medium.—The preparation of the liquid medium is similar to that of agar, except that the agar solution is replaced by 850 c.c. of distilled water. In the absence of agar, calcium is not present in a sufficient concentration to produce a precipitate with the phosphate present in the medium. There is, therefore, no necessity to heat the medium at an alkaline pH during the preparation.

This medium, whether solid or liquid, is a suitable substitute for meat infusion agar and broth for general purposes. It will support the growth of all bacteria of medical interest which have been tested and are able to grow on meat media.

STANDARDISATION OF MEDIA

While many bacteria show vigorous growth within a fairly wide range of acidity or alkalinity, there are others which require the reaction of the medium to be adjusted within narrow limits before multiplication takes place. Moreover, all organisms have a particular reaction at which the growth is optimal.

In order, therefore, to secure the best growth, particularly of the highly parasitic organisms, it is necessary that the adjustment of the reaction should be made as accurately as possible.

Two methods are in use—the one in which the acidity or alkalinity is expressed in terms of the absolute acidity which depends on the hydrogen-ion concentration ; the other, in terms of the neutral point of an indicator (phenolphthalein) is referred to as Eyre's method. The former method is the more satisfactory and is now almost exclusively used.

Standardisation according to Hydrogen-ion Concentration. — The true acidity of any fluid depends on the number of dissociated hydrogen ions present, and the reaction of the medium is dependent on, and measured by, the hydrogen-ion concentration. The greater the concentration, the more acid the medium.

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[1] Glutamine, although rapidly destroyed by heat in watery solution, appears to be stable to autoclaving in CCY agar.

Even a typically neutral liquid such as pure water undergoes dissociation (though extremely slight) into hydrogen and hydroxyl ions. It can easily be shown (*a*) that the hydrogen and hydroxyl ions are equal in number and exactly balance and neutralise each other; (*b*) that the greater part of the water is undissociated (un-ionised); (*c*) that an equilibrium exists between the ions and un-ionised water; and (*d*) that, at equilibrium, the product of the concentrations of the hydrogen and hydroxyl ions is a constant, which is termed the ionisation constant (or dissociation constant). From conductivity measurements it has been found that the concentration of the hydrogen ions (and therefore of the hydroxyl ions) in pure water is 10^{-7}. This means that in one litre of pure neutral water there is $1/10^7$ gram of hydrogen ions and the equivalent weight of hydroxyl ions. If an acid, *e.g.* hydrochloric acid, is now added, it dissociates liberating hydrogen ions, the amount of which depends on the amount of acid added and the degree to which it dissociates. The hydrogen-ion concentration is accordingly increased, while to maintain equilibrium, the number of hydroxyl ions is proportionately reduced. When an alkali, *e.g.* sodium hydroxide, is dissolved in water, it undergoes ionisation with the production of hydroxyl ions, the amount of these being proportional to the amount of alkali and its degree of ionisation, and a corresponding decrease in hydrogen ions occurs. However alkaline the solution may be, there will still be some hydrogen ions left, in such proportion that the ionisation constant remains unchanged. It will be seen, therefore, in spite of the fact that a solution may be alkaline, its reaction can still be expressed in terms of the hydrogen ions present, the stronger the alkali the smaller the concentration of the hydrogen ions.

It is, however, inconvenient to express acidity, for example, as 2×10^{-6}, or alkalinity as $1{\cdot}5 \times 10^{-9}$ grams of hydrogen ions per litre, and to simplify the matter the pH or hydrogen exponent scale has been introduced.

The pH value of a liquid is defined as the logarithm of the reciprocal of the hydrogen-ion concentration. Thus:

$$\text{pH} \propto \frac{1}{\text{concentration of H-ions}} \propto \frac{1}{\text{acidity}}$$

For neutral water where the concentration of hydrogen ions is 10^{-7} grams per litre the pH is $\log \frac{1}{10^{-7}} = 7$. In the two examples quoted above, for the acid the $\text{pH} = \log\frac{1}{2 \times 10^{-6}} = 6 - \log 2 = 5{\cdot}699$, while for the alkali the $\text{pH} = \log\frac{1}{1{\cdot}5 \times 10^{-9}} = 8{\cdot}824$.

Since this is a logarithmic scale a change of one unit in pH is equivalent to a tenfold change of hydrogen-ion concentration, that is a tenfold change of acidity; thus, a liquid of pH 5 is ten times more acid than one of pH 6, while a liquid of pH 9 is ten times more alkaline than one of pH 8. It will also be seen that as the pH depends on the *reciprocal* of the hydrogen-ion concentration, the lower the pH number the greater will be the acidity. As the neutral value is pH 7, a pH value of less than this number indicates an acid solution, and greater than this an alkaline solution.[1]

The pH value of blood plasma is about 7·5, that is, it is slightly alkaline. This reaction is about the optimum for the growth of most pathogenic organisms.

It is not practicable to use the hydrogen electrode for general laboratory use, and a simple and satisfactory colorimetric method has been devised whereby media can be adjusted to any desired pH. The method depends on the fact that when a fluid contains buffer salts such as phosphates, and an indicator, the addition of alkali or acid does not cause an abrupt change in the colour of the indicator. For example, phenol sulphone-phthalein (phenol red) is yellow in acid solution and purplish pink in alkaline solution. If an alkali be added gradually to an acid phosphate solution containing phenol red, the change in colour will commence at pH 6·8, and the colour will become more purplish pink, until the final change is reached at pH 8·4; thus the "range" is pH 6·8—pH 8·4, and as it covers the optimum reaction for culture media—namely, pH 7·4 to 7·8—this particular indicator is used.

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[1] N/10 HCl has an approximate pH value of 1
N/100 HCl ,, ,, ,, 2

Other dyes have their own definite range of pH in which colour change occurs, and there is now available a complete series of indicators which exhibit colour changes between pH 1 and pH 11 as follows:—

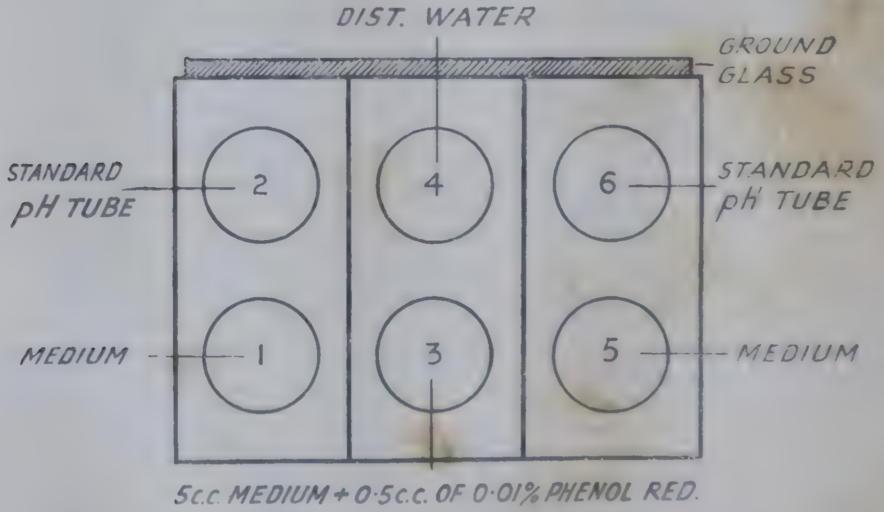
Indicator.	*Range of* pH.	*Colour change.*
Thymol blue (acid range)	1·2–2·8	red to yellow.
Bromo-phenol blue . .	2·8–4·6	yellow to violet.
Bromo-cresol green . .	3·6–5·2	yellow to blue.
Methyl red . . .	4·4–6·2	red to yellow.
Bromo-cresol purple .	5·2–6·8	yellow to violet.
Bromo-thymol blue .	6·0–7·6	yellow to blue.
Phenol red . . .	6·8–8·4	yellow to purple-pink.
Cresol red . . .	7·2–8·8	yellow to violet-red.
Thymol blue (alkaline range) . . .	8·0–9·6	yellow to blue.
Cresolphthalein . .	8·2–9·8	colourless to red.
Phenolphthalein . .	8·3–10·0	colourless to red.
Thymolphthalein . .	9·3–10·5	colourless to blue.
B.D.H. "Universal" .	3·0–11·0	red—orange—yellow—green—blue—reddish violet.

The procedure for adjusting culture media to a definite pH is comparatively simple. Solutions of fixed and known hydrogen-ion concentrations are prepared and to each solution a definite amount of indicator (for this purpose phenol red) is added. The resulting tint is the standard to which the medium must be brought by titration with alkali, and so the amount of alkali to be added per litre may easily be calculated.

Apparatus required :

1. A set of tubes of standard bore containing buffer solutions of known pH to which the indicator has been added. These solutions are made up by mixing N/15 Na_2HPO_4 and N/15 KH_2PO_4 in certain proportions. The tubes with the standard solutions, which have a range of pH 6·6–8·0 at intervals of pH 0·2, can be purchased.[1] Details of their preparation may be found in larger works.
2. A special comparator rack.
3. " Cordite " tubes, which have a uniform thickness of wall and bore, and are identical with the tubes containing the standard solutions.

PLAN OF COMPARATOR RACK


DIST. WATER
GROUND GLASS
STANDARD pH TUBE
2
4
6
STANDARD pH TUBE
MEDIUM
1
3
5
MEDIUM
5c.c. MEDIUM + 0·5c.c. OF 0·01% PHENOL RED.

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[1] These can be obtained from British Drug Houses, Ltd.

4. A solution of phenol red, 0·01 per cent.,[1] in distilled water.

5. N/20 NaOH made up as follows :—

500 c.c. N/10 NaOH
91 c.c. 0·01 per cent. phenol red
distilled water to 1000 c.c.

6. A burette, preferably a microburette, measuring to 0·01 c.c. (An improvised one may be made from a 1 c.c. graduated pipette, a short piece of rubber tubing, a glass tube drawn out to a fine point, and a pinchcock.)

Tube 3 contains 5 c.c. of the medium + 0·5 c.c. of 0·01 per cent. solution of phenol red.

Tubes 1 and 5 contain the medium only.

Tube 4 contains distilled water only.

Tubes 2 and 6 are the standard tubes for comparison. By this arrangement of the tubes the colours of 1 and 2, and of 5 and 6 are superimposed when examined in the rack.

It has been found easier to bring the solution to a tint midway between two standard colours than to make the tint match a given standard. Thus, suppose a reaction of pH 7·5 is required, then standard tubes pH 7·4 and pH 7·6 are placed in positions 2 and 6 of the rack.

The N/20 NaOH solution is run into tube 3 until the tint produced is midway between the tints of tubes 2 and 6, and the amount noted. The average of two readings is taken and the calculation is as follows :—

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[1] First prepare a stock 0·02 per cent. solution as follows :—weigh out 0·1 gram phenol red, add to this 10 c.c. (accurately) of N/10 NaOH and 20 c.c. of distilled water. Dissolve by gentle heat. Transfer the contents to a 500 c.c. volumetric flask, washing out all the indicator into the flask. Now add accurately 10 c.c. N/10 HCl, and fill up to the mark. The 0·01 per cent. solution of phenol red is made by diluting the stock solution with an equal part of distilled water.

Let the number of c.c. of N/20 NaOH = y.

5 c.c. medium require y c.c. N/20 NaOH to adjust reaction to pH 7·5.
1000 c.c. medium require $200y$ c.c. N/20 NaOH.
1000 c.c. medium require $10y$ c.c. N/1 NaOH.

Example: Suppose $y=1{\cdot}15$, then 11·5 c.c. N/1 NaOH are required per litre of the medium to adjust the reaction to pH 7·5.

It will readily be seen that the tint due to the mixture of the medium and the indicator in tube 3 is compensated for by the medium in tubes 1 and 5. The indicator is incorporated in the standard alkali solution, so that when the medium in tube 3 is titrated, the actual concentration of the dye always remains constant.

It is preferable when making media in bulk to have the reaction distinctly alkaline, and to adjust it for use by the addition of acid. The addition of alkali to an acid medium causes a precipitation of phosphates and the medium has to be filtered again before use. If, however, the medium be made distinctly alkaline, acid has to be added to obtain the correct reaction ; no precipitate occurs and the medium is perfectly clear. In this case the titration with the standard pH tubes is carried out in exactly the same manner, except that, instead of caustic soda solution, N/20 HCl containing the indicator is employed, and the calculated amount of normal hydrochloric acid is added per litre to obtain the desired reaction.

The standardisation of a solid medium such as nutrient agar presents greater difficulty than in the case of fluid media. The medium may be titrated when liquid, but the exact determination is not easy to obtain with any degree of accuracy. It has been found that agar of good quality has very little effect on the reaction of the broth to which it is added, but the reaction of the finished agar should be controlled by titrating the melted medium and then comparing the colour when cold. We have found the following method satisfactory : Mix together 0·5 c.c. of the melted agar, 4·5 c.c. of hot neutral distilled water and

0·5 c.c. of 0·01 per cent. phenol red solution; cool and compare with the standard tubes. Gelatin may conveniently be adjusted if the medium is liquefied and kept at about 37° C.

Other indicators used for bacteriological work are bromo-cresol-purple, which changes colour from yellow to violet over the range pH 5·2–6·8, and bromo-thymol-blue, which has a colour range from yellow to blue between pH 6·0–7·6 (*vide* p. 113).

Lovibond Comparator.[1]—This instrument is very convenient for estimating the pH of culture media. The indicator is added to a tube of medium and the colour is compared with that of a series of standard coloured glasses corresponding to various pH values. In matching colours the natural tint of the medium is compensated for by viewing the colour of the glass with a tube of the medium placed behind it.

DETERMINATION OF pH VALUES OF BACTERIAL CULTURES

A knowledge of the pH of bacterial cultures and of the pH changes which they undergo during cultivation is often of importance and is sometimes of practical value (*e.g.* in the differentiation of *Streptococcus agalactiae* from *Streptococcus pyogenes*). Accurate determinations of pH values can be obtained by use of the glass or hydrogen electrode, but, for general purposes, the colorimetric methods already described (*vide* p. 114) are suitable. However, in the case of bacterial cultures often only small quantities of the fluid are available and it becomes necessary to use the capillator for the pH determinations. The " B.D.H. Capillator Outfit "[2] is the best method and is available with indicators and cards to cover a range from pH 1·2–pH 11·0 (see p. 113).

The technique is as follows: The pH is first approximately determined by the use of a universal indicator. Such an indicator is a mixture of indicators which operate over a wide range of pH (*e.g.* the B.D.H. Universal Indicator—range pH 3–pH 11; or better, an indicator such as the B.D.H. " Four-

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[1] Obtainable from British Drug Houses, Ltd.

[2] For full details see catalogue, British Drug Houses, Ltd.

Eleven," which is now available in capillator sets, and has a range pH 4–pH 11). A list of the colours which may be obtained, and the corresponding pH values are supplied with the indicator. A small quantity of the bacterial culture is withdrawn with a sterile capillary pipette and transferred to a white tile and the appropriate amount of indicator added. From the resulting colour of the mixture the approximate value of the pH is obtained; for example, pH 4. The tile is appropriately sterilised after use in a 3 per cent. lysol solution.

The pH is then determined more accurately, using a capillator and choosing an indicator which operates over the desired range; for the example given above bromo-cresol green (pH range 3·6–5·2) would be chosen. The capillator consists of a series of capillary tubes filled with buffer solutions containing an indicator. These tubes show the colours corresponding to different pH values over the whole range of the indicator, and the pH value corresponding to each colour is marked on the card.

The determination of the pH is carried out by mixing together equal quantities of the indicator and culture, and then matching against the colour standards. A capillary tube, identical in size with those in the capillator, is fitted with a teat and is used for withdrawing the indicator, which is pipetted on to a tile or small watch glass. The same "pipette" is used for withdrawing an equal amount of culture and the two fluids are mixed on the tile or watch glass, sucked back into the capillary tube, and the resulting colour matched against the standards and the pH value thus obtained.

Errors due to the colour of the medium itself can be corrected by using a compensating cell. Care should be taken when working with pathogenic cultures, and the used capillary tubes should be dropped into lysol.

PEPTONE WATER

This is a simple medium, consisting of

Peptone 1 per cent.
Sodium chloride 0·5 per cent.

dissolved in warm water and then filtered. It is sterilised in the autoclave. It is used chiefly as the basis for sugar fermentation media, since broth and nutrient agar, being made from meat, may contain a small amount of muscle sugar, and it is essential that the basic medium, to which various carbohydrates are added for fermentation tests, should be free from natural sugar.

Peptone water is best made up in bulk and distributed in 250 c.c. amounts in 10 oz. screw-capped bottles. The caps are tightly screwed on and the whole batch of bottles is sterilised in the autoclave at 5 lbs. pressure for thirty minutes. The peptone water is later distributed into tubes or small bottles as required.

Plain peptone water *without indicator* is distinguished by a WHITE bead in the container, while peptone water *with indicator* is identified by a BROWN bead.

Peptone water is used to test the formation of indole (*vide* p. 425), and also for the enrichment of the *Vibrio cholerae*, when isolating this organism from infected material. In the latter case the medium should be adjusted to a reaction neutral to phenolphthalein (approximately pH 8·4), as *V. cholerae* grows better in an alkaline medium.

PEPTONE WATER AGAR.—This consists of peptone water solidified with 2 per cent. agar, and is used as a basis for solid media containing sugars (*vide* p. 333).

SUGAR MEDIA

Under the designation of "sugars" are included a variety of fermentable substances, chiefly carbohydrates, which are used in the identification and classification of organisms. These are fermented with the formation of acid, and in many cases gas is formed in addition.

The substances most commonly employed are the following :—

Monosaccharides :
- (a) *Pentoses*—
  - (1) Arabinose (from gum acacia ; and from sugar beet boiled with dilute sulphuric acid).
  - (2) Xylose (from corn cobs boiled with dilute acid).
  - (3) Rhamnose, obtained by hydrolysis of quercitin (from dyer's oak).

(b) *Hexoses*—
  - (1) Glucose (Dextrose or grape sugar).
  - (2) Laevulose (from many plants; formed in the inversion of cane sugar).
  - (3) Mannose (from the ivory nut).
  - (4) Galactose (made by the hydrolysis of lactose).

Disaccharides:
  - (1) Saccharose (Sucrose or cane sugar).
  - (2) Maltose (Malt Sugar).
  - (3) Lactose (Milk Sugar).
  - (4) Trehalose (from ergot and several species of yeasts and fungi).

Trisaccharide:

Raffinose (from cotton-seed meal and sugar beet residues).

Polysaccharides:
  - (1) Starch (soluble starch is usually prepared from potato starch).
  - (2) Inulin (from dahlia tubers).
  - (3) Dextrin (made by the partial hydrolysis of starch).
  - (4) Glycogen (from the livers of mammals and lower animals; occurs also in yeasts and certain fungi).

Alcohols:
  - (a) *Trihydric.*—Glycerol (Glycerin; from hydrolysis of fats).
  - (b) *Tetrahydric.*—Erythritol (Erythrite; from *Protococcus vulgaris*, also present in many lichens).
  - (c) *Pentahydric.*—Adonitol (Adonite; from *Adonis vernalis*).
  - (d) *Hexahydric*—
    - (1) Mannitol (Mannite; from manna).
    - (2) Dulcitol (Dulcite; from dulcitol-manna and various plants).
    - (3) Sorbitol (Sorbite), produced from dextrose by treatment with hydrogen under pressure.

Glucosides (vegetable products which on hydrolysis yield a sugar):
  - (1) Salicin (from the bark and leaves of some willows and poplars).
  - (2) Coniferin (from coniferous woods and asparagus).
  - (3) Aesculin (from the inner bark of the horse-chestnut tree).

Non-carbohydrate Substance:

Inositol (Inosite)—a benzene derivative; widely distributed in plants; extracted from walnut leaves and mistletoe.

The medium consists of peptone water, to which the fermentable substance is added in the proportion of 0·5 or 1 per cent. An indicator is incorporated to detect acid change. This may be Kubel-Tiemann litmus solution (now rarely used), neutral red (0·25 per cent. of a 1 per cent. solution), Andrade's indicator (1 per cent.),[1] or phenol red (about 1·5–2 per cent. of a 0·02 per cent. solution). If acid is produced, the litmus turns bright red; the neutral red, pink; Andrade's indicator, reddish pink; and phenol red, yellow. In order to detect gas, a small inverted tube is placed in each culture tube (Durham's fermentation tube). During the process of sterilisation the heat drives out the air from the inverted tubes, which when cool should be completely filled with liquid and contain no air bubbles.

The original method of making the medium is as follows:—

The stoppered test-tubes containing the small inverted tubes are sterilised by dry heat in the hot-air oven. The peptone water (with the indicator added) is sterilised by autoclaving. The sugars are made up separately in 10 per cent. solutions in distilled water, which are sterilised in the steamer or by filtration. The requisite amount of sugar solution is added to the peptone water. The medium is tubed (*vide* p. 159), and steamed for twenty minutes on three successive days.

The various sugar media in tubes can be distinguished by having the cotton-wool stoppers of different colours. It is better to employ wool dyed in bulk rather than to colour white-wool stoppers with various stains (p. 155).

The following alternative method of making sugar media is recommended:

Peptone water with an indicator is tubed, the Durham fermentation tube inserted, and the test-tubes stoppered with coloured cotton-wool. They are then sterilised in the steam steriliser, or in the autoclave for half-an-hour at 5 lbs. pressure. The sugars are made up separately in 10 per cent. solutions in distilled water, and are sterilised, preferably by filtration (Seitz filter), or in the steamer. The sterile sugars are kept conveniently in 10 oz. screw-neck bottles fitted with a siphon and hooded pipette, as shown in the diagram (p. 123).

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[1] Made by adding N/1 sodium hydrate to a 0·5 per cent. solution of acid fuchsin until the colour just becomes yellow.

This method of obtaining small quantities of sterile fluid from bulk can be applied to serum as well as to sugars (*vide* p. 127).

The apparatus recommended consists of a special chromium-plated metal fitting adapted to a 10 oz. bottle with a screw neck. It consists of a straight piece of tube (A), with a curved side arm (B); around this is a slightly wider tube (C), with a side arm (D), and fitted to a screw cap (E) which screws on to the bottle, a rubber washer (F) ensuring an air-tight joint. To the upper end of tube (A) is connected a Seitz filter by means of a short piece of pressure tubing furnished with a screw clamp, while attached to the lower end, by means of a short piece of rubber tubing, is a glass tube, 5 mm. in diameter, reaching to the bottom of the bottle. To the side arm (B) is connected a piece of rubber tubing furnished at the other end with a pinchcock and hooded pipette. The hooded pipette is closed by a coloured cotton-wool stopper[1] containing a small glass test-tube to cover the delivery tube. The side arm (D) is connected by pressure tubing to a cotton-wool air filter, the other end of which is attached to a filter pump.

The whole apparatus, as figured (p. 123), is connected up (the joints being bound by copper wire), wrapped in kraft paper, and sterilised in the autoclave.

Filtration of the sugar is accomplished in the usual manner, and before the pressure is released, the pressure tubing to the Seitz filter is closed by means of the screw clamp. The filter is then removed, and the end of the pressure tubing plugged with a piece of glass rod. The filter pump is now disconnected, when the air pressure forces the solution down the siphon tube as far as the pinchcock, so that the siphon is in operation as soon as the pinchcock is opened. Alternatively, air may be forced through the cotton-wool filter on D by means of a rubber blowball to start the siphon action. In use, the neck of the bottle is held by a clamp at the top of a tall retort stand. The stem of the hooded pipette is held below the bottle by means of another clamp, at a height convenient for placing a test-tube under it to receive the sugar solution. The coloured-wool stopper is removed and the inside of the pipette flamed. After use, the stopper is replaced and the hooded pipette fastened to the neck of the bottle by a piece of copper wire. The number of drops per c.c. delivered from the pipette is determined, so that the amount of sugar solution required for the volume of peptone water is easily estimated. Thus, if a pipette delivers 18 drops per c.c., then 9 drops (0·5 c.c.) of 10 per cent.

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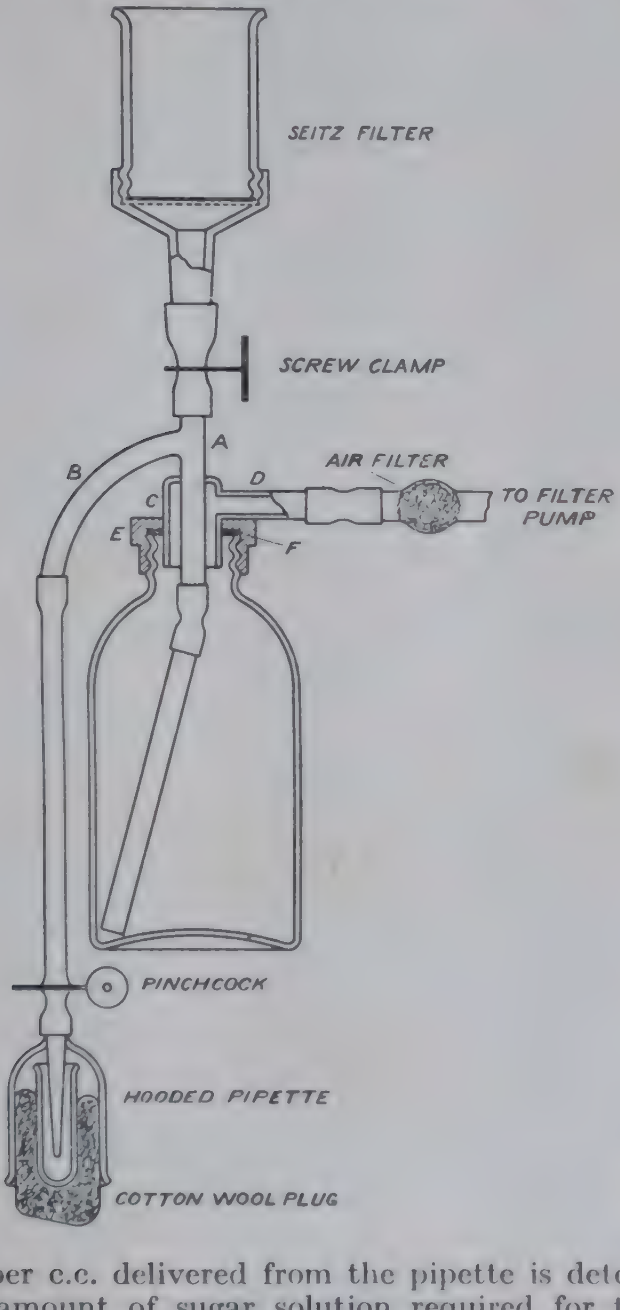
[1] The colour denotes the particular sugar used.


SEITZ FILTER
SCREW CLAMP
A
B
AIR FILTER
D
C
TO FILTER PUMP
E
F
PINCHCOCK
HOODED PIPETTE
COTTON WOOL PLUG

sugar solution per tube of 5 c.c. peptone water gives a final concentration of 1 per cent. sugar.

Expensive sugars should be made up in 10 c.c. amounts of a 10 per cent. solution in ½ oz. screw-capped bottles and sterilised by submerging the whole bottle including cap in a water-bath at 60° C. for one hour. This procedure we have found satisfactory and obviates the loss occasioned by the filtration method (*vide* method of withdrawal on p. 129).

It is recommended that sugar media as above be distributed in 5 c.c. amounts into small screw-capped bottles (p. 97), instead of test-tubes. By this means the medium can be stored without risk of contamination or alteration in the concentration of the ingredients. This is particularly useful in the case of certain sugars which are only occasionally required. Moreover, sugar media in these bottles can easily be transported without leakage or spilling. As a result of shaking during transit, air may enter the Durham fermentation tube, but it is easily removed. The bottle is merely inverted, the Durham tube drops into the neck of the bottle, and the amount of fluid is such that the open end of the tube is below the surface. The bubble of air escapes and on turning the bottle to the proper position the Durham tube falls to the bottom of the bottle full of liquid and without any air.

A considerable saving in space and material is effected by using smaller screw-capped bottles, ¼ oz. capacity and a 19 mm. Durham tube. 3 c.c. of medium only is required and the results are as satisfactory as with the larger bottles.

When the bottle has been inoculated the cap should be *loosely* screwed on to allow access of air.

In order to identify the various sugar media the caps are painted with a cellulose paint such as "Luc," of which many colours are available. The screw caps, as received from the makers, are already sprayed white, and the added colours on the tops of the small screw-capped bottles indicate the same sugars as the coloured cotton-wool plugs. Thus, in the London County Council laboratories, green = glucose, red = lactose, mauve = mannitol, etc. (p. 156).

After the bottles have been prepared, the appropriate colour is painted on the caps. A batch of a gross takes only a few minutes, and the paint is dry within thirty minutes. When two colours are used together for identifying media—*e.g.* yellow and white for inulin—only one half of the white cap is painted.

LITMUS MILK

Used in testing for the fermentation of lactose and clotting of milk.

Fresh milk is steamed for twenty minutes and then allowed to stand for twenty-four hours in order that the cream may separate. The milk is siphoned off and litmus is added in the proportion of 2½ per cent. of an alcoholic solution.[1] The medium is distributed in 5 c.c. amounts in screw-capped bottles or tubes and then sterilised by steaming for twenty minutes each day on three successive days. If bulk amounts (*e.g.* 250 c.c.) are put up it is advisable not to add the litmus solution until the milk is redistributed in smaller amounts, as the colour fades on storing.

SERUM AND BLOOD MEDIA

These may be divided into two classes:

(1) Where the medium consists almost entirely of serum or blood, which can be coagulated by heat (above 60° C.) so that a solid medium results.

(2) Where the serum or blood is added in fluid form to enrich simpler media.

MEDIA CONSISTING ALMOST ENTIRELY OF SERUM OR BLOOD

LÖFFLER'S BLOOD SERUM.—To ox, sheep or horse serum is added one-third of its volume of 1 per cent. glucose-broth. The mixture is added to stoppered sterilised tubes which are laid on a sloped tray and placed in the serum inspissator. The temperature is then slowly raised to 75° C. and maintained for six hours, when the serum coagulates to a yellowish-white solid. The tubes are thereafter sterilised at 90° C. (in the top of the steam steriliser) for twenty minutes on each of three successive days. If sterile serum is used, only two hours' inspissation at 85° C. is necessary. Further heating is detrimental, and overheating causes expansion of air bubbles and the formation of steam from the fluid droplets in the partially solidified material, which leads to disruption of the medium.

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[1] *Litmus solution.*—Litmus granules 80 grams, 40 per cent. industrial spirit 300 c.c. Grind up the granules and place in a flask with 150 c.c. of the spirit and boil for one minute. Decant the fluid and add remainder of spirit to the granules; then boil for one minute. Decant the fluid and add to the first quantity of the extract. Make up to 300 c.c. with 40 per cent. spirit and add N/1 HCl drop by drop, shaking continuously till the fluid becomes purple. To test for correct reaction, take a tube of tap water and one of distilled water, boil both and add one drop of the solution to each; the tap water should be blue and the distilled water mauve.

Löffler's medium is best made up in the small screw-capped bottles (1 oz. or ¼ oz.). The requisite number of bottles fitted with caps (as received from the makers) are autoclaved at 15 lbs. pressure for twenty minutes. The sterile serum-glucose-broth mixture is added to the bottles in 5 c.c. or 2 c.c. amounts, according to the size of bottle, under sterile conditions. The caps are then tightly screwed on, and the bottles carefully laid in a slightly sloping position in the inspissator. The temperature is slowly raised to 85° C. and maintained for two hours. The culture medium should be allowed to cool before being handled.

In these containers Löffler's serum is most useful for diphtheria diagnosis in school-clinics, small hospitals, etc., where cultures are made only from time to time. The medium can be stored for long periods, and the small amount of water of condensation present keeps the surface constantly moist. A very profuse growth occurs after incubation for a few hours. The caps should only be loosely screwed on during incubation. The use of the smaller bottles is most economical.

Löffler's serum is especially useful for the growth of the diphtheria bacillus. Not only does it produce a luxuriant growth in a short time (twelve to eighteen hours), but it is also valuable in eliciting the characteristic staining reaction of the organism by Neisser's method (*vide* p. 374).

Collection of Blood.—A sterile wide-mouthed stoppered bottle is taken to the abattoir at a time when animals, preferably sheep, are being killed. After the neck vessels have been severed, the blood is allowed to flow for a short time and then the stream from the carotid artery is allowed to spurt directly into the bottle. When filled, the bottle is stoppered and returned carefully to the laboratory. The clot is then separated from the sides of the bottle by means of a stiff sterile wire. The blood is kept overnight in the ice-chest and the clear serum pipetted off. With care, contamination can be avoided.

Defibrinated blood is collected in a similar way. The stoppered bottle, however, contains glass beads. The bottle is only half filled, and immediately a sufficient quantity of blood has been collected the stopper is replaced and the bottle continuously shaken for about five minutes. The blood so treated does not clot on standing.

Another useful method is the following :—

Horse blood is obtained from the slaughter-house, the horse being bled directly into jars containing 10 c.c. of a 10 per cent. solution of neutral potassium oxalate per litre of blood. The red corpuscles are allowed to settle overnight in the cold and the plasma is siphoned off into a Winchester quart bottle ; 22·5 c.c. of a 4 per cent. solution of calcium chloride per litre of plasma are added, and the bottle is shaken immediately on a machine until the fibrin has separated. (The plasma coagulates more quickly and fibrin separates more easily if it is warmed to room or body temperature before the calcium chloride is added.) The serum is now filtered through a Seitz bacterial filter (14 cm. diameter disk) into a large sterile screw-capped bottle of 1–5 litres capacity, fitted with siphon delivery tube and hooded pipette, as described on p. 122. The serum is stored in the cold-chamber and used as required.

Sterile specimens can be obtained by inserting a cannula or wide-bore needle into the external jugular vein. If a sheep is selected, the wool is clipped from the side of the neck and the part shaved. Contamination can be minimised by placing a bag made of waterproof material over the head of the animal. It is best to use a cannula connected by rubber tubing to a screw-capped bottle (*vide* p. 97), the whole being enclosed in kraft paper and sterilised. The vein may be made prominent by pressure on the lower part of the side of the neck. The skin over the vein is carefully sterilised with soap and water and then alcohol. The cannula is inserted into the vein and the requisite amount of blood removed. Horses are treated similarly except that it is advisable to make a small incision with a sharp knife in the skin over the vein. The cannula is then more easily introduced.

The sterile defibrinated horse blood is immediately distributed in 10 c.c. amounts into sterile ½ oz. screw-capped bottles and stored in the refrigerator. In smaller laboratories amounts of 5 c.c. will probably suffice. The defibrinated blood thus stored will keep for periods up to three months.

Inspissator.—This apparatus is used for the preparation of Löffler's serum medium, and Dorset's egg medium (*vide infra*). It consists of a water-jacketed copper box, the temperature of which can be regulated automatically. The serum or egg medium is tubed and placed in special racks, so that the tubes are at the correct angle for forming slopes. The temperature used is generally 75° C.. At this temperature the protein material is completely solidified, but the temperature is not so high as to cause bubbles of steam to disrupt the surface of the medium. As the medium is apt to dry if kept in the inspissator for any time, a small opening should be present in the inner wall communicating with the top of the water-chamber above the level of the water. Water-vapour can enter the interior of the inspissator and the medium is kept moist. Electric inspissators without a water-jacket do not yield such satisfactory media if tubes with cotton-wool stoppers are used.

HISS'S SERUM-WATER. — As certain pathogenic organisms — *e.g.* streptococci, pneumococcus — will not grow well in ordinary sugar media, it is necessary for fermentation tests to use a medium containing serum.

One part of serum is mixed with three parts of distilled water, and 1 per cent. of Andrade's indicator is added. Some samples of horse serum may give fallacious results and batches should be tested before use. Sheep or ox serum is suitable. (Some workers prefer to substitute 0·1 per cent. peptone water for the distilled water.) The various sugars are incorporated in the proportion of 1 per cent. This medium, if not acid, does not coagulate on heating, and may be sterilised in the steamer in the same way as other sugar media—namely, twenty minutes each day on three successive days.

Alternatively, the mixture of serum, distilled water and indicator is sterilised in the steamer, and the appropriate amount of the requisite sugar is added as described on p. 121.

Some workers prefer to use phenol red as the indicator, and to adjust the reaction of the medium to a suitable pH—*e.g.* 7·6—before sterilisation. In this case the medium is pink in colour and changes to yellow when acid is formed.

Fermentation is indicated by the production of acid, which alters the indicator and causes coagulation of the medium.

Small screw-capped bottles may be used most conveniently as containers for this medium. The method recommended is similar to that for peptone-water sugar media, p. 124.

The Hiss's serum water is made up with indicator, but without any carbohydrate added. The medium is distributed in 2·5 c.c. amounts in ¼ oz. bottles. The caps are tightly screwed on, and the whole batch is sterilised in the steamer for twenty minutes on three consecutive days. When a batch of any particular carbohydrate medium is required, the requisite amount of sterile 10 per cent. sugar is added from the siphon-filter bottle described on p. 122. The caps are then painted with " Luc " cellulose paint according to the sugar used (*vide* p. 156). Alternatively, small quantities of the rarer sugars in 10 per cent. aqueous solutions may be sterilised (p. 124) and stored in ½ oz. screw-capped bottles fitted with a perforated cap and red rubber washer similar to the blood-culture bottle described on p. 184. When the sugar solution is required it is withdrawn from the bottle by perforating the rubber with the needle of a sterile syringe in exactly the same manner as the rubber cap of a vaccine bottle. Contamination will not occur if the procedure is carried out carefully and under a hood (p. 167), and the medium with the sugar added keeps as long as that sterilised in a tightly closed container.

See **Appendix.**—Hiss's medium containing starch.

MEDIA ENRICHED WITH SERUM OR BLOOD

These media are used for certain delicate pathogens, such as the pneumococcus, gonococcus and meningococcus, which usually grow feebly or not at all on ordinary media.

Serum-Agar.—Ordinary nutrient agar containing

2 per cent. agar *plus* 10 per cent. of sterile uncoagulated serum. Animal serum is ordinarily used, and can be obtained in the laboratory by bleeding a rabbit (*vide infra*), allowing the blood to coagulate in a sterile measuring cylinder stoppered with cotton-wool, or in an agar-lined tube,[1] and removing the serum after it has fully separated. All the necessary precautions must be taken to avoid contamination. Serum can be stored in sealed tubes after heating for one hour at 57° C. on three successive days, but fresh serum yields much better results than heated serum in the culture of certain pathogens—*e.g.* gonococcus.

Horse serum kept in a sterile bottle, as described on p. 122, is recommended where large quantities of serum-agar are used.

The agar is first melted and then cooled to about 50° C. The serum is usually added to the agar in tubes, and, after it is incorporated, the medium is either solidified in the form of slopes, or poured into Petri dishes (*vide* p. 162). As serum-agar is indistinguishable from ordinary agar, the tube should be marked " +S " or plugged with coloured wool (blue and white). If in a small bottle, one-half of the white cap is painted blue.

Sterile hydrocele fluid or sterile ascitic fluid, withdrawn aseptically, may be used instead of serum.

SERUM-SMEARED AGAR.—This is made by running a few drops of sterile serum on the surface of an agar slope or plate (*vide* p. 162). This medium is not so satisfactory as the preceding one, but is useful in an emergency.

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[1] The agar-lined tube prevents the clot from adhering to the wall of the tube and ensures its free contraction, thus giving a large yield of serum. 1½ per cent. agar in normal saline is used and it need not be filtered. It is stored in 100 c.c. amounts in bottles and melted as required. Ten c.c. of the melted agar are added to a stoppered sterile 8 × 1 in. boiling tube, which is tilted and rotated until the agar flows all over the interior surface and sets in the form of a thin layer.

SUGAR MEDIUM FOR THE GONOCOCCUS, MENINGOCOCCUS, ETC.—

Peptone	20 grams
Sodium chloride	5 grams
Distilled water	900 c.c.

Dissolve in steamer for thirty minutes. Make alkaline to phenol phthalein and steam for a further thirty minutes. Filter through Chardin filter paper and adjust reaction to pH 7·6. Add 100 c.c. digest broth of the same pH. Add 25 grams agar powder (2·5 per cent.) and autoclave for forty-five minutes in "free steam," and fifteen minutes at 5 lbs. pressure. Filter through paper pulp, and bottle in 100 c.c. amounts. Add 5 c.c. of 0·04 per cent. phenol red solution[1] to each bottle and sterilise for one hour in "free steam" and five minutes at 5 lbs. pressure.

For use 100 c.c. of the agar is melted, cooled to 55° C., and to it is added 5 c.c. guinea-pig or rabbit serum (not horse), and 10 c.c. of 10 per cent. sterile solution of the required sugar. (This gives a concentration of 5 per cent. serum and 1 per cent. sugar.) The mixture is immediately distributed into sterile tubes or $\frac{1}{4}$ oz. bottles and tested for sterility by incubation.

The sugars generally used are glucose, lactose, saccharose and maltose.

It should be noted that when the sugar is fermented by the organism and acid is formed, the colour of the medium changes from purple-pink to yellow.

BLOOD-AGAR.—This is an important medium and is specially suitable for the gonococcus, the haemophilic group of bacteria (e.g. *B. influenzae*) and other delicate pathogens.

Either human or animal blood is suitable.

Human blood may be obtained easily by means of vein puncture (see blood culture, p. 182).

Defibrinated rabbit blood obtained from the ear vein or by cardiac puncture can be recommended for general use.

20 to 30 c.c. of blood may be obtained easily from the ear vein of a large rabbit without distress to the animal. The ear is shaved and sterilised with sterile gauze soaked in spirit. Meanwhile a small vessel containing vaseline has been heated over the Bunsen to render it sterile, and when cool, but still fluid, the

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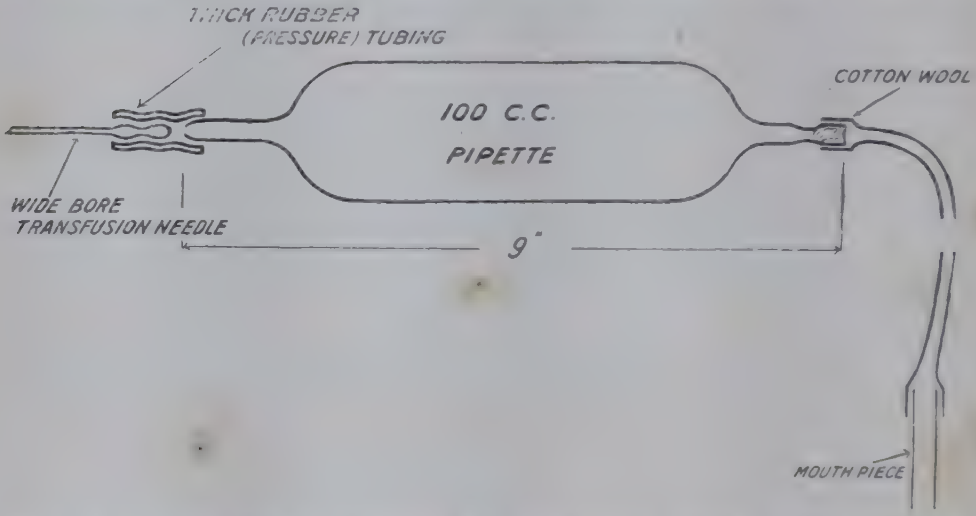
[1] Made as described in the footnote on p. 115 except that the total volume is 250 c.c.

vaseline is painted over the vein, and on the margin and under side of the ear. The ear is held forward and the vein is made prominent by means of a small spring clip at the base of the ear, and then incised with a small sharp sterile scalpel. The blood flows over the vaseline, and is allowed to drop into a sterile flask containing glass beads (*vide infra*). The vessels of the ear can be dilated by holding an electric bulb below it or by rubbing the part not covered by vaseline with a pledget of wool moistened with xylol. When sufficient blood has been obtained the clip is removed and a piece of cotton-wool pressed firmly over the cut in the vein. The xylol is removed from the ear with spirit, and some vaseline then lightly smeared on. Water should always be provided in the cage of the animal after bleeding.



This is a very useful method for obtaining small quantities of sterile rabbit blood.

For cardiac puncture the procedure is as follows :—

The animal is fastened to a board and the fur clipped over the left side of the chest ; the area is shaved and then sterilised with alcohol and ether. A 100 c.c. bulb pipette (see diagram) is cut down at both ends to 9 in. in length, one end being slightly tapered and the other end stoppered with cotton-wool. It is wrapped in kraft paper and sterilised in the hot-air chamber. A wide-bore transfusion needle is fitted into a short length (1½ in.) of thick rubber tubing and sterilised by boiling. When the animal is anaesthetised, the rubber tubing is attached to the tapered end of the pipette and to the other end is fitted a mouth-piece such as that used in pipetting (*vide* p. 253). The needle is inserted into the left side of the chest and suction applied. The needle should lie in the right ventricle of the heart, and blood rapidly flows into the pipette. About 50 c.c.

of blood per kilo. of body-weight can be obtained. The blood is then transferred to a sterile 500 c.c. flask or bottle containing glass beads. Agitation should be kept up for at least five minutes, to ensure that all the fibrin is separated.

The blood is added to melted 2 per cent. agar at 50° C. in the proportion of 5–10 per cent., as in preparing serum-agar (*q.v.*).

A considerable saving of blood can be effected if blood-agar plates are made in the following manner :—

Pour a thin layer (about 5 c.c.) of melted plain agar into a 4 in. Petri dish and allow to set. Make 10 per cent. blood-agar by adding defibrinated blood to melted agar at 55° C., and pour a similar quantity on the surface of the agar in the dish and allow it to set. Since the surface layer only is utilised for growth, blood is not required in the lower part of the dish. A fairly thick layer of medium is required to prevent excessive drying during incubation. If this were entirely 10 per cent. blood-agar, the medium would be almost opaque when viewed by transmitted light through the dish, and methaemoglobin formation by organisms would be difficult to see. Moreover, haemolysis would not be easy to determine in young cultures if a thick blood-agar layer were present. The method of the double layer of agar and blood-agar is not only more economical, but it yields a bright, light-transmitting medium on which methaemoglobin formation and haemolysis can easily be observed.

If only one plate is required, two 5 c.c. amounts of agar are melted. The contents of one are poured into the Petri dish as the first layer. The contents of the other are cooled to 55° C. and 0·5 c.c. blood added. After mixing, this is poured on the surface of the first layer in the dish and allowed to cool.

It is advisable however to prepare several plates at one time as follows :—Two 100 c.c. screw-capped bottles of agar (p. 158), 10 c.c. defibrinated horse blood in a ½ oz. screw-capped bottle (p. 128), and fourteen sterile 4 in. Petri dishes are required. The agar is melted in the steriliser and both bottles are cooled to 55° C. The contents of one bottle are distributed into the Petri dishes and the agar is allowed to set. Into the other bottle are poured the 10 c.c. of blood from the ½ oz. screw-capped bottle. No pipette is necessary as the screw-cap keeps the lip of the bottle sterile. The cap is again screwed on, the bottle inverted several times to mix thoroughly the agar and blood. The blood-agar is now distributed into the Petri dishes on the surface of the first layer of agar. Any bubbles caused by the mixing can easily be removed by drawing a Bunsen flame quickly across the surface of the medium in the dish. Two 50 c.c. amounts of agar and 5 c.c. of blood will make six plates.

For special purposes, amounts of blood up to 50 per cent. may be added.

Messrs. Burroughs Wellcome Ltd. supply sterile oxalated horse blood suitable for making blood-agar. This may conveniently be used in laboratories where there is difficulty in obtaining directly sterile animal or human blood.

HEATED-BLOOD-AGAR ("CHOCOLATE AGAR").—This medium is suitable for cultivating *B. influenzae* and certain other organisms, such as the pneumococcus. To 5 c.c. melted digest agar medium at 60° C. add 0·5 c.c. (9 or 10 drops) of defibrinated rabbit blood. Heat the mixture by immersing the tube for exactly one minute in boiling water, and allow the medium to solidify in the sloped position. If a plate is required, 12 c.c. of agar and 1·5 c.c. of blood are used.

EGG MEDIA

Dorset's Egg Medium

This medium is used for growing the tubercle bacillus. Four "new laid" eggs are beaten up and 25 c.c. distilled water then added. The mixture is strained through muslin to remove air bubbles, run into sterile tubes (*vide* p. 159), and solidified in the sloped position in the serum inspissator at 75° C. The tubes are then sterilised at 90° C. (at the top of the steam steriliser) for twenty minutes each day on three successive days.

All apparatus used should be sterile, and the eggs, before they are broken, should be placed for a few minutes in alcohol; on removal the alcohol is allowed to evaporate.

The addition of sufficient basic fuchsin to the medium to render it pale pink is advisable, as early growths of the tubercle bacillus are thus more easily seen.

As the tubercle bacillus may take some weeks to grow, the tubes are sealed after inoculation by pushing down the cotton-wool stopper below the top of the tube, and pouring in a little melted paraffin wax, or they can be covered with a wax "hermedisk."[1] We strongly advocate, however, that all media for growth of the tubercle bacillus be distributed into 1 oz. screw-capped bottles as described on p. 161.

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[1] Supplied by G. T. Gurr, London.

A useful *modification of Dorset's medium* is the following :—

Break into a sterile bowl fresh eggs which have been washed in soap and water and then dried, and beat thoroughly with a sterile knife to mix the yolks and whites. Strain the mixture through sterile cheese-cloth over a filter funnel, and to every 75 c.c. of egg mixture (usually two eggs) add 25 c.c. of sterile digest broth and 1 c.c. of a 1 per cent. aqueous solution of crystal violet. Tube the medium in small sterile bottles (avoiding the formation of air bubbles) and coagulate in a slightly sloped position in the inspissator at 75° C.. Sterilise by heating in the inspissator at 75° C. for two hours the next day. The material to be inoculated should be well rubbed over the surface of the medium.

GLYCEROL-EGG MEDIUM (for growing the human type of tubercle bacillus) is prepared as above, but with the addition of 5 per cent. of glycerol to the digest broth and egg mixture.

Löwenstein-Jensen Medium for the Cultivation and Differentiation of Human and Bovine Types of the Tubercle Bacillus

(1) *Mineral Salt Solution.*[1]

Potassium dihydrogen phosphate KH_2PO_4 (Analar)	0·4 per cent.
Magnesium sulphate (Analar) . .	0·04 ,,
Magnesium citrate	0·1 ,,
Asparagin	0·6 ,,
Glycerol (Analar)	2·0 ,,

in distilled water.

Heat to dissolve.

The solution is boiled or placed in the steamer for two hours and allowed to cool overnight. 600 c.c. is a convenient quantity to prepare.

(2) *Salt-Starch Solution.*

To each 600 c.c. are now added 30 grams of potato starch.[1] This is mixed and heated in a water-bath with constant stirring for fifteen to twenty minutes, until a satisfactory paste is produced, and then allowed to remain for one hour in a water-bath at 56° C..

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[1] The chemicals used by us are obtained from British Drug Houses, Ltd., and where specified, the Analar (Analytical Reagent) product should be used.

(3) *Egg Fluid.*

Hens' eggs are washed, to disinfect them thoroughly, in a 5 per cent. soft-soap solution, after which they are left in running water for one to two hours. The eggs must be less than one week old, and 20 eggs are usually sufficient for 1 litre of egg fluid, but if they are small, 22 eggs should be used. The eggs are now broken one by one into a sterile cup, the yolk and white mixed, and poured into a sterile vessel.

For each 600 c.c. of salt-starch solution, 1 litre of egg fluid is required. The egg fluid is mixed very thoroughly for ten minutes (or shaken in a machine), after which it is filtered through sterile gauze, and to it is added the salt-starch solution at the termination of one hour in the water-bath at 56° C..

(4) *Malachite Green Solution.*

Make a 2 per cent. solution of malachite green in distilled water and place in the incubator for one to two hours. To each 1600 c.c. of prepared substance (1 litre egg fluid + 600 c.c. salt-starch solution) add 20 c.c. of 2 per cent. malachite green.

The medium is now tubed in 5 c.c. amounts in 1 oz. bottles (p. 161) and the caps are tightly screwed on. The bottles are laid horizontally in the inspissator and are heated at 75° C. for half-an-hour. They are allowed to remain in the inspissator overnight, and are heated again the following day at 75° C. for half-an-hour.

In the screw-capped bottles the medium will keep for many months, but if slopes are made in test-tubes they must be stored in the cold and used within a month.

On this medium the human type of tubercle bacillus grows very luxuriantly, in the form of large heaped-up dry yellow colonies, while the bovine type shows small discrete colourless colonies.

This medium will show good primary growth of tubercle bacilli (*e.g.* from sputum after treatment with 4 per cent. caustic soda, *vide* p. 401) in ten to twelve days. It can be strongly recommended, particularly for the human type.

POTATO MEDIA

Large potatoes are selected, carefully washed and peeled. By means of a potato borer (or large cork borer) a cylinder of potato is obtained, which is then washed in running water to remove excess of starch. This is cut obliquely and each half is placed in a test-tube with the thick end resting on a plug of cotton-wool, or in a special potato tube, or in a wide-mouth screw-capped 1 oz. bottle ("Universal container"). The tubes are then filled with sterile water and placed in the steam steriliser for half-an-hour. The water is poured off and the tubes are autoclaved at 10 lbs. pressure for twenty minutes.

ALKALINE POTATO MEDIUM.—Prepared as above, but instead of filling the tubes with water 0·7 per cent. sodium bicarbonate solution is added. The subsequent treatment is the same.

GLYCEROL POTATO.—Also prepared as above, 5 per cent. solution of glycerol being used instead of water. This medium is useful for differentiating the eugonic and dysgonic types of the tubercle bacillus.

MEDIA FOR SPECIAL PURPOSES

MACCONKEY'S BILE-SALT NEUTRAL RED LACTOSE AGAR

This is a useful medium for differentiating intestinal organisms of the coli-typhoid group. It is a peptone solution solidified with agar, to which bile-salt, 0·5 per cent., and lactose, 1 per cent., are added, with neutral red as the indicator.

Peptone, 2 per cent., and sodium taurocholate (commercial), 0·5 per cent. are dissolved by heat in tap water. Then add 2 per cent. agar and dissolve in the steamer or autoclave. Clear with white of egg (*vide* p. 104) and filter. (Large quantities may be filtered through paper pulp in the same way as agar—*vide* p. 105.) Add a sufficient amount (about 0·6 c.c. per 100 c.c.) of a freshly prepared 1 per cent. watery solution of neutral red to give the medium a distinct reddish-brown colour. If the medium is acid, and assumes a rose-pink colour, add caustic soda solution until the colour becomes definitely reddish brown. (It is preferable to adjust the reaction beforehand to pH 7·6 which gives the correct colour with neutral red.) The medium is then sterilised in the steamer and finally 1 per cent. lactose (previously sterilised separately in a 10 per cent. watery solution) is incorporated. The completed medium may be sterilised as in the case of other sugar media.

Organisms which produce acid from lactose—e.g. *B. coli*—form rose-pink coloured colonies, whereas the colonies of non-lactose-fermenters—e.g. *B. typhosus*—are colourless.

MacConkey's medium immediately after being filtered should be bottled in 100 c.c. and 250 c.c. amounts in 4 oz. and 10 oz. screw-capped "round bottles." It is then sterilised as above. The 100 c.c. of media when melted will be sufficient for seven Petri plates, while 250 c.c. will make eighteen.

When MacConkey's medium is stored for any length of time the neutral red indicator tends to fade. In order to overcome this, the medium is made up as follows without neutral red, and the pH is adjusted so that the correct shade of colour is obtained when the indicator is added.

Make the stock fluid alkaline to phenol phthalein. Add N/1 sodium hydroxide solution until a drop of the fluid, placed on a tile with a drop of phenol phthalein solution (0·5 per cent. in 50 per cent. alcohol), produces a pink colour. Filter when cold, add the agar powder, dissolve by heat and filter through paper pulp (*vide* p. 105). Keep the batch hot and add 1 per cent. of lactose powder. Take a 500 c.c. sample, add 1·5 c.c. of 2 per cent. neutral red solution (*vide infra*), and add N/1 acid until the correct tint is obtained. Calculate and add the amount of acid required for the bulk of the medium, bottle and sterilise.

The MacConkey agar without indicator is indistinguishable from ordinary nutrient agar, but is identified by a RED bead (p. 157). The neutral red is made up in a 2 per cent. solution in 50 per cent. alcohol and 0·3 c.c. per 100 c.c. of medium is the average quantity used. When plates are to be poured the bottle of MacConkey agar is melted, the indicator added, the screw-cap replaced, and the contents thoroughly mixed before pouring.

LEIFSON'S DESOXYCHOLATE CITRATE AGAR

(for the isolation of intestinal pathogens) [1]

Composition—

Pork infusion	1000 c.c.
Peptone	10 grams

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[1] For full details see Leifson, E., *J. Path. Bact.*, 1935, **40**, 581. See **Appendix** for a simpler form of this medium.

Agar	20 grams
Lactose	10 ,,
Sodium citrate ($2Na_3C_6H_5O_7$, $11H_2O$)[1] .	25 ,,
Sodium desoxycholate	5 ,,
Lead chloride (optional) (1 : 300,000) .	3·5 mgm.
Ferric ammonium citrate (green scales) .	2 grams
Neutral red (1 : 50,000) . . .	20 mgm.
pH–7·4	

Preparation.—To fresh, lean, ground pork add three times its weight of distilled water and allow the whole to infuse for about an hour. The lumps of meat should be broken up and distributed in the water. Then add 1 c.c. of N/1 HCl for each 100 grams of meat and cook the mixture for about one minute. Strain off the meat and filter the infusion through paper until clear and free from visible fat. To this add the same quantity of N/1 NaOH as was added of HCl. Again boil for one minute and filter through paper. Adjust the volume to that of the water originally added. From 100 grams of meat is thus obtained 300 c.c. of infusion.

To the infusion so prepared add 1 per cent. of peptone and sufficient NaOH to make the pH about 7·5. Boil for two to three minutes and filter through paper. Add 2 per cent. of agar and 0·5 c.c. of N/1 NaOH for each 100 c.c. of medium. Allow the agar to soak for at least fifteen minutes and melt by boiling or in the autoclave. To the melted agar add the other ingredients, down to lead chloride in the order given, and as rapidly as possible. If the medium is not to be poured immediately it should be stored at this composition. The volume should be noted carefully and written on the storage bottles.

To this medium, melted and at a temperature of 80°–100° C., add 0·2 per cent. of ferric ammonium citrate. Titrate to pH 7·4, using a phenol red indicator (certain indicators, as bromo-thymol blue, do not give the correct pH) and add the neutral red. 0·2 c.c. of a 1 per cent. solution of neutral red is required per 100 c.c. of medium. Care should be taken to keep the temperature of the medium to which the iron and neutral red are added sufficiently high to kill all vegetative cells of bacteria, which may also get in. If desired, the medium may be titrated to pH 7·4 before the addition of the iron, and a neutralised solution of iron added. Pour the finished medium immediately into Petri dishes, about 15 c.c. to each. It should be protected as much as possible from light and drying.

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[1] The sodium citrate should have a known formula, and if different from that given, should be added in equivalent molecular concentration.

The use of Leifson's desoxycholate-citrate-agar for the isolation of intestinal pathogens.—This medium has proved useful for isolation of the typhoid bacillus, Flexner type of dysentery bacillus, paratyphoid bacilli, *B. suipestifer*, and many others. Some strains of paratyphoid bacilli are inhibited, as also the Shiga, Sonne, *dispar* and *alkalescens* types of dysentery bacilli. Cholera vibrios grow poorly. *B. pyocyaneus* grows well. *B. alkaligenes* usually does not grow. *B. proteus* is variable, some strains are inhibited to some extent, but most strains are not. Most strains of colon bacilli are inhibited to a large extent. Gram-positive bacteria of all kinds are inhibited completely. Various types of yeasts and moulds will grow.

The colon bacilli produce red colonies. If there is lead in the medium the bacilli which produce H_2S give brown-centred colonies. If proteose and like peptones are used, *B. typhosus* and *B. paratyphosus A* generally produce transparent colonies, all other bacteria opaque colonies. Colonies of *B. pyocyaneus* may have a slightly greenish tinge, but they are not easy to recognise. Colonies of *B. proteus* do not spread. Owing to the inhibiting effect of the medium on the normal intestinal flora, large amounts of faecal material may be inoculated on the medium. It is best to incubate the plates for at least twenty hours to allow the *B. coli* colonies time to develop their characteristic red colour.

ENDO'S MEDIUM (ROBINSON AND RETTGER'S MODIFICATION)

Distilled water, 1 litre ; agar, 25 grams ; beef extract (Lab. Lemco), 5 grams ; peptone, 10 grams. Add the water to the agar, reserving 10 per cent. to dissolve the other ingredients. Dissolve the agar by heating in the autoclave at 15 lbs. for thirty minutes. Dissolve the peptone and meat extract by heating in the water-bath. Combine the two parts and make up to 1 litre. Adjust to pH 8·5 with 10 per cent. sodium carbonate solution, or adjust first to pH 7 and then add 10 c.c. of 10 per cent. sodium carbonate. Filter when hot. Bottle and sterilise in the autoclave.

When required for use melt the agar, and to each 100 c.c. add 10 c.c. 10 per cent. solution of lactose, 0·26 c.c. saturated alcoholic solution of basic fuchsin, and 1 c.c. 10 per cent. solution of sodium bisulphite.

Colonies of *B. typhosus* are greyish white, those of *B. coli* are red.

WILSON AND BLAIR'S BISMUTH SULPHITE MEDIUM

(for the isolation of typhoid and paratyphoid bacilli)[1]

Prepare a stock bismuth-sulphite-glucose-phosphate mixture as follows :—

Dissolve 30 grams bismuth-ammonio-citrate scales in 250 c.c. boiling distilled water. Add to this a solution obtained by boiling 100 grams anhydrous sodium sulphite in 500 c.c. distilled water, and then while the mixture is boiling add 100 grams sodium phosphate crystals (Na_2HPO_4, $12H_2O$). To the bismuth-sulphite-phosphate mixture when cool add a solution of glucose obtained by dissolving 50 grams of commercial glucose in 250 c.c. boiling distilled water. This mixture will keep for months.

Prepare an iron-citrate-brilliant-green mixture consisting of—

1 per cent. solution of iron citrate scales (ferric citrate scales) in distilled water . . .	200 c.c.
1 per cent. brilliant green in distilled water . .	25 c.c.

This mixture will keep for months.

Make up the medium as follows :—

Nutrient agar, 3 per cent. (melted and cooled to 60° C.)	100 c.c.
Stock bismuth-sulphite-phosphate-glucose mixture	20 c.c.
Iron-citrate-brilliant-green mixture . . .	4·5 c.c.

Pour into Petri dishes.

The use of this medium depends on the property of *B. typhosus* to reduce the sulphite to sulphide in the presence of glucose, and the inhibition of *B. coli* by brilliant green and by bismuth sulphite in the presence of an excess of sodium sulphite. Isolated colonies of *B. typhosus* and *B. paratyphosus* are black, the former usually appearing within twenty-four hours and the latter within forty-eight hours.

TELLURITE TRYPSIN COPPER SULPHATE SERUM AGAR

(for the isolation of the diphtheria bacillus)

Several media containing compounds of tellurium have been devised for the isolation of *B. diphtheriae* from mixed cultures, and the following modification of Douglas's medium by Allison and Ayling[1] gives excellent results.

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[1] Wilson, W. J., *J. Hygiene*, 1938, **38**, 507, and personal communication.

Reagents required :

(*a*) 2 per cent. nutrient agar (pH 8) with a basis of either (1) Hartley's broth, or (2) meat extract + 1 per cent. peptone.[2]

(*b*) Solution of copper sulphate (Analar)[3] 10 per cent. in distilled water.

(*c*) Sterile tellurite trypsinised serum prepared as follows :—

Horse serum	100 c.c.
Liquor Trypsin Co.[4]	6 c.c.
Potassium tellurite[3] aqueous solution 2 per cent.	10 c.c.

In the preparation of the sterile tellurite trypsinised serum, the serum is drawn from horse blood which has been allowed to clot. Liquor Trypsin Co. and a 2 per cent. solution of potassium tellurite are added in the proportions given above and allowed to stand at refrigerator temperature for twenty-four hours. The mixture is then filtered through a Seitz bacterial filter into a sterile container fitted with a siphon delivery tube and hooded pipette, and stored for use as required (*vide* diagram, p. 123) ; for small quantities the mixture may be distributed in amounts of 10 c.c. in sterile bottles and stored in the refrigerator. It is important that the horse serum be used fresh and without the addition of chloroform.

To each 100 c.c. of nutrient agar, melted and cooled to 50° C., are added 10 c.c. of the sterile trypsinised serum tellurite mixture, and 0·5 c.c. of the 10 per cent. copper sulphate solution. After thorough mixing the medium is poured into sterile Petri dishes (about 14 c.c. in a plate of 4 in. diameter) and dried in the incubator. The medium is perfectly transparent and keeps well.

An alternative method which works well is as follows :—

Trypsinised serum	10 c.c.
Copper-tellurite mixture	2 c.c.
Nutrient agar, pH 8	100 c.c.

The copper-tellurite mixture is made up of equal parts of 2 per cent. copper sulphate (Analar) and 2 per cent. potassium tellurite.

The advantage possessed by this medium is that it completely inhibits nose and throat organisms—such as staphylococci, streptococci, *D. catarrhalis*, pneumococcus, pneumobacillus, etc., while the diphtheria bacillus, Hofmann's bacillus and organisms of the diphtheroid group grow unrestricted, with typical colony appearances; moreover the spread of organisms of the *B. proteus* type, so often present in ear swabs, is inhibited.

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[1] Allison, V. D., and Ayling, T. H., *J. Path. Bact.*, 1929, **32**, 299.

[2] Parke, Davis & Co.

[3] British Drug Houses, Ltd.

[4] Allen & Hanbury.

B. diphtheriae grows typically as a round smooth colony with a dark centre well demarcated from a greyish-white periphery ; another type frequently met with is a tiny round greyish colony with a smooth glistening surface and no blackening. Colonies of Hofmann's bacillus grow as round glistening smooth colonies with a large grey or black central zone fading to a very thin grey periphery, and are lighter in colour than colonies of *B. diphtheriae*, from which they are readily distinguishable. Organisms of the diphtheroid group grow luxuriantly as large colonies, round, smooth and glistening, but their predominant characteristic is a dark brown coloration of the central zone as compared with colonies of the diphtheria bacillus. Other organisms which occasionally appear in cultures are : *B. proteus*, which grows as a large grey raised glistening colony showing little or no tendency to spread ; yeasts, which grow as dull greyish-white colonies, and a Gram-positive tetracoccus, which appears as a dark brown rugose colony. These colonies are readily distinguishable from those of the diphtheria bacillus and the diphtheroid group. A low-power binocular magnifier (10–20 diameters) is of value in examining cultures.

Plates of the medium may be inoculated directly from swabs or from cultures on Löffler slopes, and it should be noted that forty-eight hours' incubation of the plates is, as a rule, necessary for the naked-eye recognition of colonies and the successful use of the medium.

McLEOD'S MEDIUM (for the diphtheria bacillus)[1]

This medium consists essentially of heated-blood-agar (chocolate agar) containing 0·04 per cent. of potassium tellurite. It differs from other culture media for the diphtheria bacillus in that the meat extract which it contains is never heated above 75° C. and is sterilised by filtration.

Add 1½ to 2 lbs. of minced meat to 1000 c.c. tap water at 48° C. and keep at this temperature for one hour. Squeeze out the juice through lint or muslin, leave this in the ice-chest overnight and filter through filter paper.

To 1000 c.c. filtrate add 20 grams peptone (Parke, Davis & Co.) and 5 grams sodium chloride ; warm at 45° C. until dissolved.

In order to adjust the reaction, take 50 c.c. and heat it to

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[1] See Anderson, J., Happold, F., McLeod, J. W., and Thomson, J., *J. Path. Bact.*, 1931, **34**, 667.

80°–90° C. for fifteen minutes. Filter through paper. Determine the amount of N/10 NaOH required to bring 10 c.c. to pH 7·6 in the usual way. Add to the bulk of the fluid an amount of alkali calculated on the basis of this titration.

Filter through a Seitz K clarifying disk.

Refilter this filtrate through a Chamberland candle previously sterilised in the autoclave.

Distribute into flasks and tubes. One or two tubes should be incubated for three days at 30° C. to control sterility. The remainder should be stored in the cold until required.

Mix equal parts of this broth and melted 5 per cent. agar in water.

Add 7–10 per cent. of freshly drawn defibrinated rabbit blood, and 0·04 per cent. of potassium tellurite.

Mix and heat at 75° C. for ten to fifteen minutes before pouring into Petri dishes.

CLAUBERG'S MEDIUM

(for the diphtheria bacillus) [1]

PREPARATION OF STOCK MATERIALS.

1. *Glycerinated blood.*—Sterile glycerol (B.D.H. Analar), 1 part. Sterile defibrinated ox blood, 2 parts. Keep for six weeks in refrigerator to ripen.

2. *Dye solutions.*—(*a*) Make up a 2 per cent. solution of water blue [2] 6B extra P (Grübler) in distilled water.

(*b*) Add 2 grams of metachrome yellow, II R.D. "W" (Grübler) [3] to 100 c.c. of distilled water.

Allow to stand for two days with frequent shaking, and then filter. The filtrate is used and should be a clear deep reddish-yellow colour.

3. *Cystine solution.*—Dissolve 1 gram of anhydrous sodium carbonate (B.D.H. Analar) in 10 c.c. of boiling distilled water, and 1 gram of cystine (B.D.H.). Boil again and make up to 100 c.c. with distilled water. The solution should be clear.

4. *Potassium tellurite solution.*—Powder finely potassium tellurite (B.D.H.) in a small mortar. Dissolve 2·5 grams in 240 c.c. of distilled water which has been previously sterilised in a flask (250 c.c.) or 10 oz. bottle. Add the powder slowly and shake the flask, to ensure complete solution, and make up the volume to 250 c.c. with sterile distilled water. The solution

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[1] See *J. Path. Bact.*, 1937, **45**, 325.

[2] Methyl blue can be substituted.

[3] Chrome fast yellow G. can be substituted (obtainable from Clayton Aniline Co. Ltd., Manchester).

is clear and should not be used after six months, nor should the chemical be used after ten months.

5. *Placenta agar.*—Mince three human placentas and add to 4 litres of water, heat to boiling, and then allow to simmer for thirty minutes. Filter through gauze.

To 4 litres of this filtrate add :

Peptone, Witte's	40 grams
Sodium chloride	12 ,,
Sodium dihydrogen phosphate NaH_2PO_4 (B.P.)	8 ,,

Warm to 45° C. to dissolve. Standardise to pH 7·2. Heat in the steam steriliser for fifteen minutes. Filter through paper and check the pH of the filtrate. Add 160 grams agar and heat to dissolve. Check the pH and store in bottles. Check the pH again before use.

PREPARATION OF THE MEDIUM.

6. *Fresh sterile defibrinated ox blood* is required for each batch of medium.

Agar (see 5).—Melt in the steamer, cool to 48° C., and place in a water-bath at 48° C. until required.

7. *Blood tellurite mixture.*—In a 1000 c.c. flask place 165 c.c. of sterile distilled water and 82·5 c.c. of sterile ox blood. Allow to stand until lysis is complete, then add 11 c.c. of glycerinated blood (see 1) and 18 c.c. of potassium tellurite solution (see 4). Place the mixture in the water-bath at 48° C..

8. *Indicator solution.*—In a 100 c.c. flask place 0·75 gram of sodium acetate (B.D.H. Analar), 7·5 grams glucose (B.D.H. Analar), 30 c.c. of water blue solution (see 2 (*a*)), 10 c.c. of metachrome yellow solution[1] (see 2 (*b*)) and 5 c.c. of cystine solution (see 3). Place in the water-bath at 48° C..

Pour the indicator solution (see 8) into the flask containing the blood tellurite mixture (see 7). Mix the contents of the flask thoroughly and add 210 c.c. of melted agar (see 5). Mix again and pour plates. The heating at 48° C. should be as short as possible, as the tellurite undergoes a change when heated in the presence of glucose. Each batch of the medium amounts to 531 c.c.

Colonies of *B. diphtheriae* are blue, with marginal coloration. Colonies of *B. hofmanni* and *B. xerosis* are yellow and turn black.

TULLOCH'S MODIFICATION OF CLAUBERG'S MEDIUM [2, 3]

1. *Placenta agar.*—This is the same as Clauberg agar (see 5 above). (Hopkin and Williams peptone or other British-made peptone is used.)

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[1] See footnote 3, p. 144.

[2] Personal communication.

[3] All reagents obtainable in this country.

2. *Short tryptic digest.*—Serum—ox or sheep—is heated to 37° C. and 10 per cent. of Liquor Trypsin Co. (Allen and Hanbury) is added, and the mixture incubated for one hour. This is filtered through a Berkefeld filter and stored in convenient amounts in bottles or tubes. This short tryptic digest is heated to 60° C. for thirty minutes on three successive days, to stop digestion and ensure sterility.

3. *Method of making up the medium.*—Placenta agar (see 1), 40 c.c., is melted, cooled to 60° C. and 30 c.c. short tryptic digest (see 2) added. The mixture is held at about 50° C., when the following are added :—

Glucose, 75 per cent.	1·6 c.c.
Fused sodium acetate, 15 per cent. solution	0·8 c.c.
Glycerinated blood (Clauberg, No. 1)	1·7 c.c.
Potassium tellurite	2·7 c.c.
Methyl blue B.D.H., 2 per cent.	4·5 c.c.
Cystine, 1 per cent. in 0·1 per cent. anhydrous sodium carbonate solution	0·8 c.c.

The medium is poured into plates at once.

A similar medium is made, but containing saccharose instead of glucose: two grams of saccharose are dissolved in 2 c.c. sterile water and added to the mixture. If the saccharose is heated more than is necessary, it tends to hydrolyse. Plates of the glucose and saccharose media are used simultaneously. Typical blue colonies indicate fermentation of the sugar. The diphtheria bacillus ferments glucose but not saccharose. Organisms which ferment both sugars, or neither, are not diphtheria bacilli.

NEILL'S MEDIUM

(for the diphtheria bacillus) [1]

PREPARATION OF MEDIUM.

1. *Broth.*—The best results are to be obtained by mixing the broth base with the plain agar just before pouring into plates.

Lemco	20 grams
" Difco proteose " peptone	20 ,,
Sodium chloride	10 ,,
Distilled water	1000 c.c.

Dissolve by steaming in the usual way. Adjust reaction with N/1 NaOH till alkaline to phenol phthalein. Heat for thirty minutes at 90° C. to bring down phosphates. Filter and bring to pH 7·6 with N/1 HCl. Bottle in 100 c.c. amounts in 6 oz. screw-capped bottles and sterilise by autoclaving for fifteen minutes at 15 lbs. pressure.

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[1] For full details see Neill, G. A. W., 1937, *J. Hygiene*, **37**, 552.

2. *Agar*—

" Difco bacto " agar [1] . . .	30 grams
Distilled water	1000 c.c.

Dissolve the agar by boiling, bottle in 100 c.c. amounts in 10 oz. bottles. Sterilise in the autoclave.

3. *Laked blood mixture.*—A litre screw-capped bottle containing 10 grams of sodium citrate, dissolved in 10 c.c. of water, is sterilised in the autoclave and taken to the slaughter house. When an ox is killed, a litre of blood is caught in a sterile funnel and is directed into the bottle, which is vigorously shaken. To the blood is added 1·25 c.c. pure formalin and 30 c.c. of methyl ether. The preparation is stored in 250 c.c. amounts in 10 oz. bottles.

4. *Potassium tellurite* (1 per cent. solution).

A bottle containing 100 c.c. of the broth is placed in a water-bath at 55° C.. 10 c.c. of the laked blood mixture and 4 c.c. of the potassium tellurite (1 per cent. solution) are added and the whole well mixed. 100 c.c. of the agar are melted by immersing the bottle in boiling water, and the melted agar is cooled to 55° C.. The broth-blood-tellurite mixture is now added to the 100 c.c. of agar in the large bottle and mixed by inverting it gently several times. The agar mixture is now heated by gradually raising the temperature of the water-bath to 75° C., at which temperature it is maintained for fifteen minutes. The medium is now ready for pouring into plates. These plates show a finely grained chocolate appearance if the medium has been properly made.

To obtain the best results, several points require emphasis.

(1) The broth should be heated above 100° C. only on one occasion—hence the mixing of the broth with the agar just prior to pouring the plates.

(2) The blood and tellurite should be added to the broth and mixed before the broth, etc., is mixed with the agar. This ensures uniform mixing and prevents the formation of bubbles and froth.

(3) The blood-broth mixture should be added *to the agar* (which must be in a large enough bottle to accommodate the whole) and should be mixed by inverting the bottle slowly

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[1] Other good quality agar may be used.

several times. This, again, prevents the formation of froth and lumps in the agar.

The technique above described gives about 210 c.c. of medium for about 12 plates.

The growth and colony characteristics of *B. diphtheriae* are similar to those on McLeod's medium, and *gravis*, *mitis* and *intermedius* types can readily be distinguished.

B. diphtheriae gravis.—Eighteen to twenty-four hours: medium small, discrete, irregular colonies, with "nippled" or conical centre, somewhat crenated edge, grey in colour, darker towards the centre, size 2 to 4 mm. in diameter.

Thirty-six to forty-eight hours: large, slate-grey coloured colonies, well separated specimens often reaching a size of 5 to 7 mm. in diameter, showing a central nipple, radial striations, irregular outline and a granular frosted surface, so aptly described as "daisy head."

Compared with appearances on McLeod's medium, the colonies are slightly larger in size and greyer in colour; this is probably due to the lower concentration of tellurite.

B. diphtheriae mitis.—Eighteen to twenty-four hours: medium small, discrete, regular colonies, spherical or lenticular in shape, colour typical, being grey at the periphery, shading to black at the centre, size 1 to 3 mm. in diameter.

Thirty-six to forty-eight hours: large, grey-black or black colonies varying in size up to 5 mm. in diameter. In form they may be either spherical or slightly conical, the outline is regular and sometimes slightly hazy, the surface is shiny, reflecting light freely, and the colour shades from a greyish colour at the periphery to a dense black at the centre.

B. diphtheriae intermedius.—Eighteen to twenty-four hours: small, metallic grey, discrete colonies, irregular in outline, edge somewhat crenated, with central papilla showing a characteristic "punched-out" appearance, size about 1 mm. in diameter.

Thirty-six to forty-eight hours: small, grey-black granular colonies about 1 to 3 mm. in diameter, irregular edge, whole appearance very rough with central papilla. These colonies have a peculiar "cut-out" appearance (not unlike pieces of confetti) which is very characteristic.

HOYLE'S[1] MODIFICATION OF NEILL'S MEDIUM

1.	Lab. Lemco	10 grams
	Peptone ("Difco" proteose or Evans) .	10 ,,

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[1] *Lancet*, 1941, i, 175. See also Appendix.

Sodium chloride	5 grams
Agar	20 ,,
Water	1000 c.c.

Adjust to pH 7·8 and autoclave. Bottle in 200 c.c. quantities in screw-capped bottles.

2. Sterile horse blood laked by freezing and thawing four times. Store in the cold, preferably frozen. See also **Appendix.**

3. Potassium tellurite	0·7 gram
Water	20 c.c.

Store tightly corked and in the dark.

To each 200 c.c. of agar, melted and cooled to 50° C., add 10 c.c. of laked blood and 2 c.c. of the tellurite solution. Pour plates.

Good growth occurs after 18–24 hours' incubation. Colonies of *B. diphtheriae* have a characteristic slate-grey colour.

Type differentiation is similar to but not quite so good as that on McLeod's or Neill's media.

This medium is simple to make and gives good results for routine examination.

BORDET-GENGOU MEDIUM

(for Bacillus pertussis)

The following modification has given excellent results and is recommended. Clean and pare potatoes and cut them into thin slices. To 500 c.c. tap water add 250 grams potato and 9 grams sodium chloride. Boil until the potato slices fall to pieces. Make up the water lost in boiling, filter through linen, and adjust the reaction to pH 7·0.

To 1500 c.c. tap water add 60 grams agar powder to give a final concentration of 3 per cent. Dissolve by heat and add 500 c.c. of the potato extract, 20 c.c. glycerol, and 20 grams proteose peptone (Difco). Distribute in bottles and sterilise in the autoclave with " free steam " for one hour, and then raise the pressure to 5 lbs. for five minutes. Store until required.

For use, melt in the steamer for one hour and invert the bottle several times. Place in the water-bath at 55° C. for five minutes until the temperature of the agar has dropped to about 70° C.. Place an equal amount of defibrinated horse blood in the 55° C. bath for 2–3 minutes to warm slightly. Add one part of blood to one part of glycerol-potato agar. Mix thoroughly and pour plates. The plates should not be dried in the incubator, but should be stored at once in the refrigerator, when they may be used up to two weeks after preparation.

DIEUDONNÉ'S MEDIUM (Blood-alkali-agar)

A selective medium for the isolation of *V. cholerae* (p. 474). Equal parts of defibrinated ox blood and N/1 sodium hydroxide are mixed and heated for one and a half hours in the steam steriliser. At this stage the mixture is unsuitable for immediate preparation of the medium and if so used would inhibit the cholera vibrio. The blood-alkali is first subjected to repeated steaming (*e.g.* one and a half hours on eight successive days) in a flask of sufficient size that a large surface of the fluid is exposed to air, and then allowed to stand at room temperature for about ten days. In this process the volatile ammonia is removed and carbon dioxide is absorbed from the air. Three parts of the blood-alkali are then added to 7 parts of 3 per cent. agar. This product will grow *V. cholerae* abundantly while inhibiting coliform bacilli and *B. proteus*. The blood-alkali can be kept in bulk for a considerable period without loss of its selective properties. The original method was to incorporate the blood-alkali in agar immediately after its preparation and to "ripen" the finished medium in plates kept at room temperature for two days, the ammonia being removed in this way and carbon dioxide absorbed. It is more convenient, however, to "ripen" the blood-alkali in bulk so that plates can be poured ready for immediate use.

LIVER INFUSION AGAR [1]

(for the cultivation of Brucella group)

Minced fresh ox liver is pulped in a mortar and 1 lb. is mixed with 500 c.c. of distilled water, and kept in the cold for twenty-four hours. It is then steamed for one and a half hours and filtered through wire gauze (60-mesh). 20 grams washed agar, 5 grams peptone and 5 grams sodium chloride are incorporated in 500 c.c. of the liver extract with 500 c.c. distilled water added. The pH is adjusted to 7·2 at 60° C. and the medium is filtered through wire gauze (as above). It is finally placed in containers and autoclaved. The ultimate pH should be 6·6–6·8.

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[1] See I. F. Huddleson, *Brucelloses in Man and Animals*. 1939. New York, p. 13.

SMITH-NOGUCHI MEDIUM

This medium was introduced by Theobald Smith and developed by Noguchi who used it for the growth of pathogenic spirochaetes. It consists of a small piece of fresh sterile animal tissue, preferably rabbit kidney, covered with a long column of ascitic fluid on which is superimposed a layer of vaseline.

The medium is anaerobic, the function of the fresh tissue being to destroy by means of its catalase any hydrogen peroxide that might be formed by organisms, and be detrimental to their growth.

The ascitic fluid must be clear, free from bile, and of high specific gravity ; it must be sterile from the beginning, as sterilisation either by heat (57° C.) or by filtration may render it unsuitable.

Technique.—Special long narrow tubes ($8 \times \frac{1}{2}$ in.) are employed.

Eight pairs of forceps and eight pairs of scissors are sterilised. A large healthy rabbit (1500–2000 grams in weight) is anaesthetised and bled by cardiac puncture (*vide* p. 131). The abdomen is opened with strict aseptic precautions and the kidneys are removed, fresh sterile instruments being used at each stage of the operation.

The kidneys are cut up into small pieces, using separate sterile instruments for each organ. Each kidney yields eight to eleven pieces, and one piece is placed in each $8 \times \frac{1}{2}$ in. tube. Sterile ascitic fluid is run in by means of a 50 c.c. bulb pipette until the tubes are half full. Vaseline, previously sterilised by autoclaving in a 250 c.c. conical flask, is melted and added by means of a 10 c.c. pipette, forming a layer of about half-an-inch. The tubes are then incubated for forty-eight hours and examined for contamination.

The tubes are inoculated by first melting the vaseline and then introducing the inoculum to the bottom of the tube by means of a capillary pipette and rubber teat. Material is similarly withdrawn to be examined. Growth is indicated by a clouding of the fluid at the bottom of the tube, which appears about the fourth to the tenth day of incubation. Turbidity, however, is not definite evidence of growth, as uninoculated control tubes also may show clouding just above the piece of tissue.

N.N.N. (NOVY, MACNEAL, NICOLLE) MEDIUM

For the growth of trypanosomes and leishmaniae.

Meat extract is made in the ordinary way with rabbit or beef flesh, using 125 grams per litre of water. To this are added peptone, 20 grams; sodium chloride, 5 grams; agar fibre, 20 grams; and 10 c.c. normal sodium carbonate solution. After tubing, autoclaving and cooling to 50° C., there is added to the medium in each tube twice its volume of defibrinated rabbit blood (see p. 131). The contents of the tubes are mixed by rotation between the palms of the hands and allowed to solidify in the sloped position, preferably on ice. It is important to obtain a large amount of water of condensation.

Before inoculation the tube is placed in the upright position, and material is usually introduced by means of a capillary pipette. The growth of leishmaniae occurs mostly in the water of condensation.

NÖLLER'S MODIFICATION OF N.N.N. MEDIUM

Agar	25 grams.
Glucose	20 ,,
Slightly alkaline broth	1000 c.c.

Prepare, filter and distribute into test-tubes in about 2 c.c. amounts. When required for use melt the medium, and when cooled to 55° C. add an equal or double volume of defibrinated horse blood, or undefibrinated rabbit blood.

LEPTOSPIRA MEDIA

Noguchi's media.—The following media were introduced by Noguchi for the cultivation of the causal organism of Infectious jaundice (Weil's disease). They are rendered semi-solid by the addition of one-tenth of their volume of 2 per cent. nutrient agar.

(1) Rabbit serum, 2 parts; saline or Ringer's solution,[1] 6 parts; citrated rabbit plasma,[2] 1 part; neutral 2 per cent. agar, 1 part. The last is added fluid at 60° C. and the contents

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[1] Sodium chloride, 9 grams; calcium chloride, 0·25 gram; and potassium chloride, 0·42 gram, per litre.

[2] 10 c.c. of blood are allowed to drop from the ear vein of a rabbit (*vide* p. 131) into a tube containing 1 c.c. of 10 per cent. sterile sodium citrate solution to prevent clotting. The blood is then centrifuged and the clear supernatant plasma pipetted off.

of the tubes are mixed by rotation to ensure a uniform distribution. A layer of sterile liquid paraffin ½ in. deep is then placed on the surface of the medium.

(2) In a tube place 8 c.c. saline or Ringer's solution [1] at 55° C.. Add 1 c.c. melted nutrient agar. Allow 20 drops of blood from the ear vein of a rabbit to fall into the tube (*vide* p. 131). The tube is not shaken and the contents are allowed to become semi-solid without mixing. Sterility is tested by incubation at 37° C. for twenty-four hours. This medium is applicable when the organisms have been accustomed for some time to artificial cultivation.

Schüffner's Medium (*modified*).—To 1500 c.c. tap water add 1·5 grams "Difco" neopeptone or Witte's peptone and boil; then add 300 c.c. Ringer's solution and 150 c.c. Sorensen's solution (prepared by mixing 72 c.c. of M/15 Na_2HPO_4 and 28 c.c. M/15 KH_2PO_4); boil until phosphates have precipitated, cool and filter. The reaction of the medium should be between pH 6·8 and pH 7·2. Place 3 c.c. quantities in clean, new, stoppered test-tubes and autoclave for twenty minutes; then add to each 0·3 c.c. fresh guinea-pig serum which has been sterilised by filtration through L_5 Chamberland candles. Heat at 56° C. for half-an-hour and test for sterility by incubation overnight.

Fletcher's Medium (*modified*).—Several rabbits are bled and the serum separated from each with aseptic precautions. As individual animals vary considerably in the suitability of their serum for cultivation of leptospirae, it has been recommended that pooled serum should be used. It is preferable, however, that separate batches of the medium should be made from each serum, samples being tested for their growth-promoting qualities and the batch giving the best results then selected for the routine cultivation of the organism. All the necessary precautions should be taken to ensure sterility of the serum. A solution consisting of 0·2 per cent. peptone (good quality) and 0·1 per cent. sodium chloride in distilled water (adjusted to pH 7·2) is prepared, added in measured quantities to sterile screw-capped bottles and sterilised by steaming. After cooling, 10 to 20 per cent. of serum is added. The bottles are then incubated at 37° C. to test for sterility. To obtain satisfactory growths large inocula introduced with a sterile pipette should be used.

This medium may be converted to a semi-solid form, which is also suitable for cultivating leptospirae, by incorporating in the peptone solution 0·1 per cent. agar, the peptone-agar being melted by heat and cooled to 50° C. before addition of the serum.

SABOURAUD'S MEDIUM

A medium for the growth of fungi, consisting of

Peptone	1 per cent.
Maltose	4 ,,
Agar	2·3 ,,

which is made up as in the case of ordinary nutrient agar but standardised to pH 5·0–5·5.

COOKED MEAT MEDIUM

This is used for the cultivation of the sporing anaerobic bacilli. The original medium is known as "Robertson's bullock-heart medium," but the following modification of Martin and Lepper is recommended :—

500 grams of fresh bullock's heart are minced, placed in 500 c.c. of boiling N/20 caustic soda, and allowed to simmer for twenty minutes, by the end of which time the neutralisation of the lactic acid will be ensured and the pH of the liquor should be about 7·5. The liquid is drained off through a muslin filter and, whilst still hot, the minced meat is pressed in a cloth and allowed to dry partially by being spread on a cloth or filter-paper. In this condition it can be introduced into test-tubes without soiling them. Enough should be placed in each to occupy about ½ in. of the tube, and covered with 10 c.c. of 1 per cent. peptone infusion broth. The tubes must be kept in a bath of boiling water for half-an-hour to drive off any dissolved oxygen. They are then autoclaved at 120° C. for twenty minutes. The inoculum should be introduced towards the bottom of the tube in contact with the meat.

It is usual to cover the surface of the medium with a layer of sterile liquid paraffin, ½ in. deep, although this is not necessary.

It is convenient also to distribute cooked-meat medium in 10 c.c. amounts in 1 oz. bottles with perforated screw caps (pp. 174, 185) without adding liquid paraffin. This method is suitable for anaerobic cultures, and also for the preservation of stock cultures of aerobic organisms.

BOECK AND DRBOHLAV'S MEDIUM
(for the cultivation of amoebae)

Four fresh eggs are well washed with soap and water, wiped over with alcohol, and broken into a sterile bottle containing glass beads. 50 c.c. Locke's solution[1] are then added and the mixture thoroughly shaken. Test-tubes are filled with sufficient of the medium to form short slopes of 1–1½ in., and the slopes are coagulated in the inspissator at 70° C.. They are then sterilised by autoclaving. To each tube is added a mixture of 8 parts sterile Locke's solution and 1 part of sterile human serum inactivated at 55° C. for half-an-hour. The fluid should cover the solid medium to a depth of ½ in., and the tubes are then incubated to test for sterility. Instead of the serum mixture a 1 per cent. solution of crystallised egg albumin in Locke's solution, sterilised by filtration through a Seitz filter, may be substituted. The initial reaction of the medium, which varies between pH 7·2 and 7·8, does not require adjustment.

Dobell and Laidlaw have modified the medium in the following manner :—

Sterile horse serum is inspissated in the sloped position for one hour at 80° C.. It is then covered with serum or egg albumin solution as above. A small quantity of solid rice starch (sterilised in thin layers at 180° C. for one hour in the hot-air oven) is added. Amoebae grow luxuriantly in this medium, and the whole life cycle, including encystment, may be observed.

WHEY MEDIA

These media are useful for the growth of *B. acidophilus* (p. 469).

Whey Broth.—Add 10 per cent. hydrochloric acid to skimmed milk heated to 80°–90° C., in amount just sufficient to precipitate the casein. Filter through cotton-wool and adjust the pH to 6·8–7·0. Now add 0·5 per cent. peptone, autoclave at 15 lbs. pressure for fifteen minutes, and filter.

Whey Agar.—Made from whey broth by the addition of 1·5 per cent. agar.

Other media used for the cultivation of particular organisms or for obtaining special cultural reactions will be described or referred to in the appropriate sections of Part III.

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[1] Sodium chloride, 9 grams; potassium chloride, 0·42 gram; calcium chloride, 0·24 gram; sodium bicarbonate, 0·1–0·3 gram; water, 1 litre.

IDENTIFICATION OF MEDIA

It is necessary to identify a culture medium after it has been made, and as many of the media are similar in appearance, *e.g.* the various kinds of nutrient agar and the different sugar media used in fermentation tests, it is essential that there should be some simple but reliable system of identification. It has long been the custom to denote the medium contained in test-tubes by cotton-wool stoppers of different colours, but for flasks some sort of gummed label has been used. In the case of bottled media, the caps are painted in various colours to distinguish the different sugars, while coloured beads are used to identify the other types of media, thus avoiding gummed labels which become detached in the steamer when a solid medium is melted. The glass beads are the ordinary opaque beads for threading necklaces, 7 mm. in diameter, and are inexpensive to buy. (Clear glass beads are not suitable.) Before use they are boiled twice in distilled water and dried in the incubator. The appropriate bead is dropped into the bottle before filling. Owing to the convexity of the bottom of the bottle, the bead remains in one corner, and is very easily recognised no matter what type of culture medium is used. On tilting the bottle for pouring, the bead comes to rest on the shoulder and remains in this position, even when the bottle is almost completely inverted. The coloured beads can be used for identification of all kinds of media and reagents. In the case of small bottles which will not take a bead, or if beads are unobtainable, a dab of coloured cellulose paint on the side or bottom of the bottle will answer the same purpose.

It is recommended that a standard colour scheme be adopted and the following system is suggested, as it is already widely used.

Fermentation Media—" Sugars "

For tubes, coloured cotton-wool is used, and for screw-capped bottles cellulose paint such as " Luc " is painted on the cap.

Where colours are mentioned for which there is no coloured wool (*e.g.* gold, silver), a small patch of cellulose paint is placed on the tube itself.

Adonitol	Silver	Laevulose	Yellow
Aesculin	Brown	Maltose	Blue and white
Arabinose	Black and yellow	Mannitol	Mauve
Dextrin	Red and mauve	Mannose	Black and green
Dulcitol	Pink	Raffinose	Red and white
Erythritol	Black and red	Rhamnose	Black and pink

Galactose	Mauve and white	Saccharose	Blue
Glucose	Green	Salicin	Pink and white
Glycogen	Blue and yellow	Sorbitol	Black and blue
Inositol	Gold	Starch	Yellow and mauve
Inulin	Yellow and white	Trehalose	Mauve and green
Lactose	Red	Xylose	Red and green
Glycerol	Brown and white		

Other Culture Media

Other media in bulk in 4–20 oz. bottles are identified by means of a coloured bead. For smaller quantities a dab of coloured paint is placed on the cap. It should be borne in mind that it is better to use a few outstanding colours alone or in combination if necessary, rather than different shades of a colour ; thus, green, irrespective of the shade, whether it be light or dark, yellowish green or bluish green, always indicates glucose.

Culture Medium	*Colour of Bead*
Digest broth	Black
Nutrient agar made from digest broth	Black
Infusion broth	Yellow
Nutrient agar made from infusion broth	Yellow
MacConkey's medium (see note on p. 138)	Red
Peptone water without indicator	White
Peptone water with indicator	Brown
Glucose media	Green
MacConkey's fluid medium—	
Single strength	1 red spot
Double ,,	2 red spots
Sabouraud's medium	Light blue

Solutions, etc.

Distilled water	White
Normal saline (0·85 per cent.)	Dark blue
Glucose in saline	Blue and green

CHAPTER V

CULTIVATION OF MICRO-ORGANISMS
(*Continued*)

USE OF CULTURE MEDIA

ONLY general methods are described here. Special methods applicable for particular purposes are referred to in the appropriate sections—*e.g.* under special media.

STORAGE AND DISTRIBUTION OF CULTURE MEDIA

Culture medium after being made is either stored in bottles[1] in bulk, or distributed in small bottles, tubes, or Petri dishes (*vide infra*). It is convenient to store fluid media in 500 c.c. and 250 c.c. amounts in 20 oz. and 10 oz. screw-capped bottles. The bottles are sterilised with the caps tightly screwed on, so that the medium remains sterile and without evaporation. For solid media, storage in 250 c.c. and 100 c.c. amounts in 10 oz. and 4 oz. round screw-capped bottles is recommended. The 250 c.c. amounts are useful for subsequent distribution into tubes or small bottles. The 100 c.c. amounts are extremely convenient for pouring into Petri dishes—*e.g.* nutrient agar, MacConkey's medium—or when melted and cooled to 55° C. the addition of 10 c.c. defibrinated horse blood or serum will make sufficient blood-agar or serum-agar for seven plate cultures (in 4 in. Petri dishes) (*vide* p. 162).

USE OF SOLID MEDIA

For immediate use the medium is allowed to solidify in sterile stoppered test-tubes either by cooling after having been melted by heat, as in the case of agar or gelatin (*vide* p. 106), or by coagulation in an inspissator, as in the case of solidified serum or egg media (*vide* p. 125). The tubes are plugged with cotton-wool, and sterilised in the hot-air oven before the addition of the medium.

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[1] *Vide* p. 96.

Alternatively 1 oz. or $\frac{1}{4}$ oz. screw-capped bottles (p. 97) can be substituted for test-tubes.

Tubing of medium is conveniently carried out by means of a sterile 6 in. glass funnel (fixed in a burette stand) with a short length of rubber tubing and glass delivery nozzle fitted to the stem and controlled by a pinchcock. During the tubing the funnel is covered with the lid of a large sterile Petri dish to avoid aerial contamination.

The latest improved model of automatic filler devised by T. H. Ayling can be recommended for tubing media.[1] It consists of a glass funnel 7 in. in diameter, connected by rubber tubing to a metal 3-way stopcock which in turn is connected to an all-glass syringe of 15 c.c. capacity (see figure, p. 160). The syringe is of the three-piece type, but without the nozzle, and the plunger is hollow, as the head of liquid will not lift a solid glass piston. The barrel is graduated to 15 c.c. by 0·5 c.c., and the numbers are engraved so as to be readable when the syringe is vertical. The syringe is connected to the stopcock by means of a metal screw fitting. A clamp secures the lower end of the syringe. The amount of fluid delivered is determined by the adjustable screw. The action of the filler is simple. The head of medium in the funnel forces up the plunger until it is stopped by the adjustable screw. The handle of the stopcock is then turned and the syringe empties itself under the weight of the plunger. Air bubbles in the syringe are removed by first filling the apparatus and then emptying and filling the syringe two or three times, manipulating the piston by hand while this is being done. The adjustable screw is then turned to deliver the correct amount. If a smoothly working syringe is used, very little head of pressure is necessary, and the height need not be greater than 18 inches.

Once set, the accuracy of the filler is much greater than that of an ordinary pipette, while media can be tubed with greater rapidity. It works equally well with melted agar or gelatin, provided that fresh hot supplies are available, and the syringe and stopcock are washed out immediately after use.

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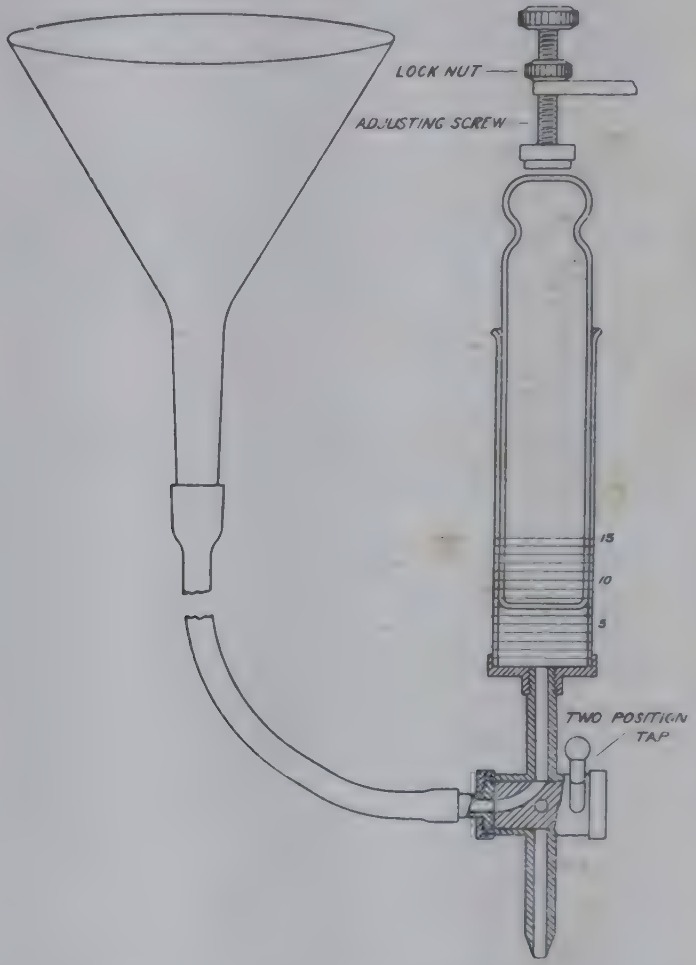
[1] Supplied by R. B. Turner & Co., London.

When tubing agar or broth with the filler, the medium is run into clean but not sterilised test-tubes. These are then plugged with cotton-wool and sterilised in the steamer or in the autoclave as indicated under the description of the various media.



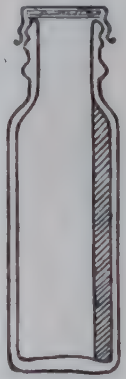
Alternatively the agar or broth is distributed into small bottles (p. 97), the caps are screwed tightly on, and the containers suitably sterilised.

Depending on the method of inoculation to be used, media are solidified in tubes as follows :—

(1) *Upright, for " puncture " or " stab " culture.*—The test-tube or small screw-capped bottle is half

filled with the medium (about 12 c.c.) which is allowed to solidify in the upright position. It is inoculated by plunging a long straight wire (*vide* p. 164), charged with the material, vertically down the centre of the tube. This method is used for anaerobic cultures in glucose-agar, and for testing the liquefaction of gelatin.

(2) *Sloped, for "stroke" culture.*—This is often called a "*slope*" or "*slant*," and ensures a maximum surface of the medium exposed to the air. Quantities of 5 c.c. of medium for ordinary $6 \times \frac{5}{8}$ in. tubes are sufficient. When a large number of agar tubes have to be sloped, special trays, which allow the tubes to be laid at the correct angle, are useful, and moreover they can be stacked one upon another so that very little bench space is required during solidification. Fresh agar slopes after cooling contain "water of condensation" at the foot of the tube, and the tubes should be stored and handled in the vertical position to prevent the fluid from flowing over the surface of the medium or entering the cotton-wool stopper.



Reproduced by permission of the "Lancet" Ltd.

1 oz. screw-capped bottles[1] can conveniently be substituted for test-tubes (see figure). The aluminium cap should have a red rubber washer 2 mm. thick. **5** c.c. amounts of the medium are added to the bottles and the caps are tightly screwed on. Instead of being sloped, the bottles are merely placed in the horizontal position during solidification (on the bench for agar, and in the inspissator for Löffler's medium and the various egg media, *q.v.*). The position of the medium is shown in the figure, the amount of 5 c.c. being just sufficient to reach the top of the shoulder. A larger surface for the same amount of medium is obtained in these bottles than in the $6 \times \frac{5}{8}$ in. test-tubes. Being

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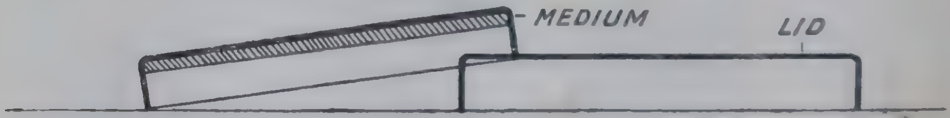
[1] These bottles, type H53, are supplied by the United Glass Bottle Manufacturers Ltd. See p. 97.

tightly sealed there is no evaporation and the surface of the medium is always moist.

Instead of 1 oz. bottles it is more economical to use $\frac{1}{4}$ oz. ("Bijou") bottles. Only 2·5 c.c. of medium (broth, agar and other solid media) are required. An extensive experience of these small bottles shows them to be very convenient for laboratory use, affording economies in media, storage and conveyance.

Plates.—Where a large surface is necessary, as in the separation of organisms from mixtures (*vide* p. 167), the medium—*e.g.* agar or gelatin—is allowed to solidify in the form of a thin layer in a Petri dish. For a dish of 4 in. diameter, 14 c.c. of medium are ample. The *melted* medium is poured into the dish with the necessary precautions to avoid contamination. Medium which has been bottled—e.g. *vide supra* (1)—can be melted and used for pouring plates.



In separating organisms in mixed cultures by spreading the material on plates, it is essential that the surface of the medium should be dry. When plates have been poured, the steam from the hot liquid condenses on the surface of the medium and this moisture is undesirable for cultural work. It is removed by drying the poured plates in the incubator at 37° C. for one hour. The lid of the dish is first laid in the incubator (see diagram); the portion containing the medium is then inverted (so that the surface of the medium is downwards) and placed in the incubator with the free edge resting on the lid. If care is taken to avoid disturbing dust, there is very little risk of contamination of the medium by air organisms.

When it is necessary to dry the surface more quickly this can be done by passing the dish containing the medium quickly several times over a Bunsen flame.

Shake Cultures.—Agar or gelatin medium in tubes—*e.g.* (1), *vide supra*—is inoculated in the melted condition at a temperature which keeps the medium fluid, but is not *immediately* lethal to the organisms inoculated (*e.g.* 45°–50° C.). The contents of the tubes are mixed by rotation between the palms of the hands and then poured at once into a Petri dish, as in water examination (*vide* p. 295), or left to solidify in the tube so that colonies may develop in the depth of the medium, as when separating anaerobes. In the latter case the test-tube is filed and broken, and the colonies "picked out" of the medium exposed in this way.

USE OF FLUID MEDIA

Fluid media are used in (1) test-tubes stoppered with cotton-wool, the tubes being about half filled; (2) $\frac{1}{4}$ oz. ("Bijou") bottles; broth or peptone water in 2·5 c.c. amounts; and fermentation media in 3 c.c. amounts; (3) 1 oz. screw-capped bottles, in 5 c.c. amounts; (4) 2 or 6 oz. screw-capped bottles for blood culture (35–100 c.c. amounts) (*vide* p. 185); or (5) stoppered or screw-capped bottles of larger capacity according to the quantity of culture required.

INOCULATION OF CULTURE MEDIA

According to the nature of the medium and the inoculum, various methods are employed for inoculation, and the following instruments are commonly used:—

"PLATINUM LOOP."—This consists of a piece of platinum wire, No. 23 S.W.G., $2\frac{1}{2}$ in. long, with one end fused into a glass rod, or inserted into a special aluminium holder. The other end of the wire is bent in the form of a loop, care being taken that the loop is flat and completely closed. Owing to the high cost of platinum, "Nichrome" or "Eureka" resistance wire No. 24 S.W.G. may be used as an efficient substitute.

The wire is sterilised by holding it vertically in a Bunsen flame so that the whole length becomes red-hot at the same time. A wire charged with certain growths—*e.g.* of the tubercle bacillus—should be sterilised slowly in the cooler part of the flame. If rapidly burned, particles of unsterilised culture may "spurt" from the wire on to the bench. The loop is the most useful of the inoculating wires. It takes up a considerable amount of solid culture, and also a large drop of fluid.

Straight Wire.—This is similar to the foregoing but without the loop. It is used for stab cultures, and also for picking off single colonies.

Long Straight Wire.—A wire 4½ in. long mounted on a holder. It is employed for deep-stab inoculation when working with anaerobes.

Thick Wire, particularly with a loop, is very useful on account of its rigidity for lifting thick viscid sputum and tenacious growths.

Wire with Lance-Head.—This is made with thick wire (*e.g.* resistance wire), one end being flattened out and filed or cut to a lance-head or diamond shape. It is employed for making scrapings from organs, for picking off tough growths—*e.g.* of the tubercle bacillus—and for disintegrating felted cultures—*e.g.* fungi.

Scalpel.—This instrument, sterilised by dipping in alcohol and flaming, is used for making inoculations with scrapings from tissues and ulcers, etc..

Sterile Capillary Pipettes.—These are made by heating the middle of a piece of quill tubing, 5 mm. bore and 8 in. long, and when melted pulling out the two halves, thus forming two pipettes. The capillary ends, which should not be too thin, are sealed in the flame, and the other ends are plugged with cotton-wool. They are placed in a large test-tube 15×2½ in., which is then stoppered with cotton-wool and sterilised by dry heat (*vide* p. 91). Before use, the tip of the capillary portion is broken off and a rubber teat fitted to the other end. These pipettes are necessary for inoculating Smith-Noguchi medium and cooked-meat medium (*q.v.*), and are very useful in many bacteriological manipulations.

Sterile Pipettes (10–100 c.c.) are used when large amounts of fluid inoculum have to be added to a medium.

Graduated pipettes are employed when measured quantities of material are used for inoculation (*vide* water examination, p. 295).

TECHNIQUE OF INOCULATING TUBES

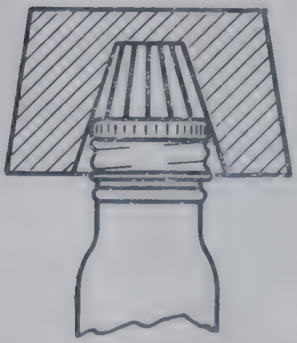
The following routine methods are recommended :—

Inoculation of one " slope " from another.—The two tubes are firmly held at their lower ends between the thumb and first two fingers of the left hand, with the sloped surface of the medium towards the worker. The tube containing the growth should be on the left and the uninoculated tube on the right. With the right hand loosen the cotton-wool stoppers by rotating them in the mouths of the tubes so that they may be removed easily. Take the holder of the inoculating wire at its end between the thumb and first two fingers of the right hand (as in holding a pen). Sterilise the wire by holding it vertically in the Bunsen flame. Remove the stopper of the tube from which the inoculation is to be made with the crooked third finger of the right hand, and flame the mouth of the tube. Pass the wire into the tube and touch a portion of the medium free from growth to ascertain if the wire is sufficiently cool. If too hot, the wire will melt the agar, causing a furrow, and might of course kill the organisms in removing the growth. When the wire is cool, the growth is scraped from the surface, care being taken not to wound the agar. Withdraw the wire, remove the stopper from the other tube with the crooked little finger and flame the mouth of the tube. Insert the wire charged with the growth and lightly smear the surface of the agar. Withdraw the wire and sterilise it, flame the mouths of the tubes and replace the stoppers. The nature of the inoculated material and also the date should be written on the tube by means of a grease pencil, or on a gummed label which is then affixed to the tube.

For *stab cultures*, the tubes are held similarly and the straight wire charged with bacterial growth is plunged into the centre of the medium, care being taken to withdraw the wire in the same line, and not to cause splitting of the medium.

In *inoculating a fluid medium*, such as broth, from a solid culture, the tube should be inclined almost to the horizontal and the growth on the loop deposited on the wall of the tube just above the surface of the liquid at the lower end of the tube. On returning the tube to the vertical position the inoculum is below the surface of the broth.

Screw-capped Bottles.—When inoculating medium in screw-capped bottles essentially the same procedure is carried out as above. Before the bottles are held in the hand it is advisable to loosen the screw-cap, as this is usually tightly screwed before sterilisation to seal the bottle effectively. If the caps are very tight they can easily be loosened by means of a bored-out rubber bung, a section of which is shown in the figure. A bung about two inches across is suitable, and by means of a cork-borer a number of holes are bored in a slanting direction round a diameter of 1¼ inches, so that the whole centre is removed, leaving a conical-shaped cavity. It is preferable to have the wall ridged, as it grips the cap more easily. The bung can be held in the hand, or suitably mounted just



below the edge of the bench. The bottles are held exactly as test-tubes, and the cap is held in the same way as the cotton-wool stopper. The bottles are then unscrewed from the cap, the wire is introduced and the inoculation made. The cap is now loosely screwed on, and when the bottle is ready for the incubator the screw-cap is tightened if considered necessary.

Inoculating Hood.—It is advisable, as far as possible, to carry out certain inoculation procedures under a hood in order to minimise the chances of aerial contamination.

A suitable size of hood is 5 ft. wide, 5 ft. deep, 7 ft. 6 in. high. It fits over the bench to form a completely enclosed chamber and is entered by a sliding (not swing) door. All sides above the bench level consist of windows. Ventilation is secured by two holes in the roof; from the top of each is attached a vent pipe 3 in. wide and 18 in. long, and turned at right angles. The bench on which the hood is fitted should have a gas supply for the Bunsen burner, and it is convenient to have a pipe from the roof 3 in. in diameter with a funnel-shaped opening situated 24 in. above the bench top, under which the Bunsen burner is placed so that the gas fumes are led directly away. The hood may be lighted by an electric lamp suspended from the roof.

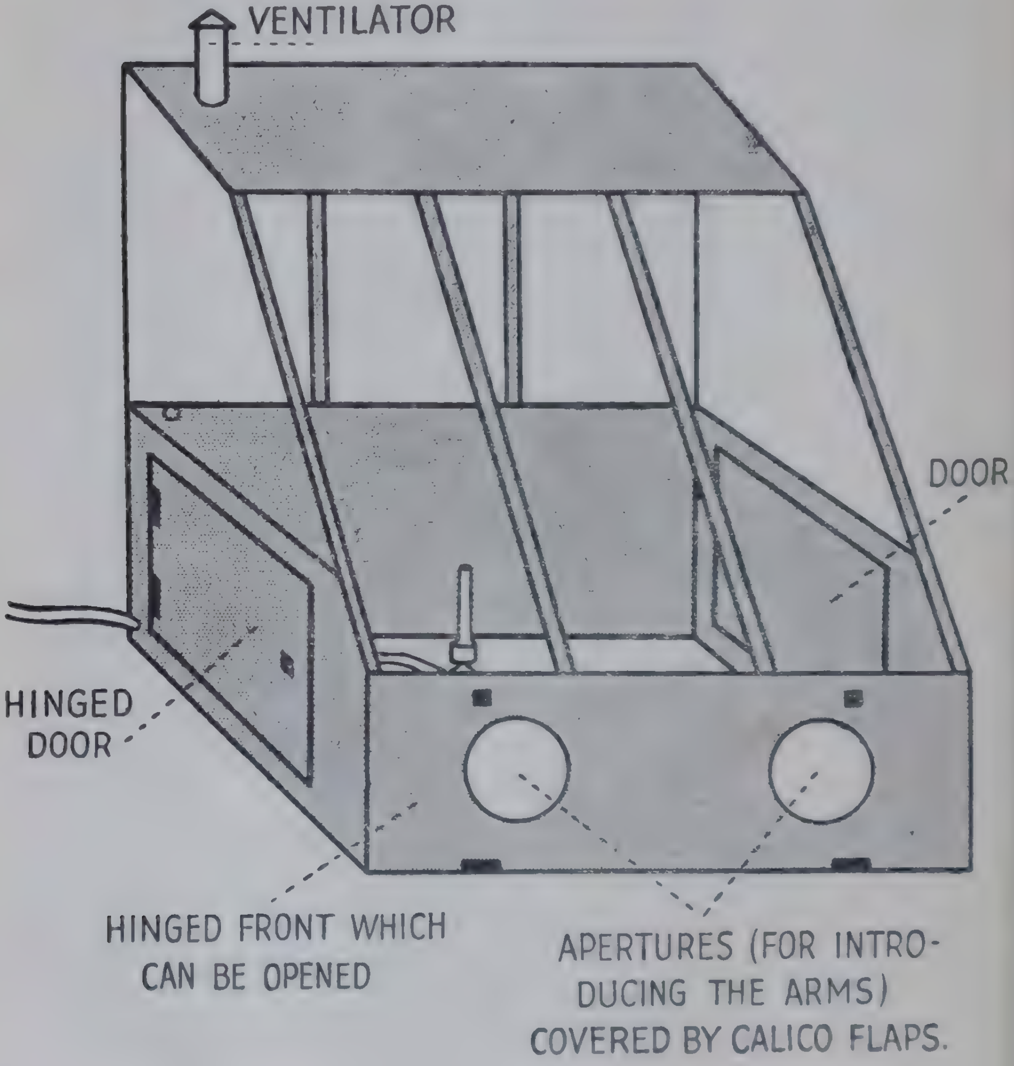
The table under the hood is covered by a towel soaked in 1 : 1000 perchloride of mercury solution, so that any organisms deposited in dust are destroyed. The advantage of the hood depends on the relative absence of dust and air currents, which are liable to produce contamination of medium, etc., exposed in the process of inoculation. We have used the inoculating hood with considerable success in the preparation of blood-agar plates and other highly nutritive media, and in conducting autopsies on animals under aseptic conditions.

A more simple inoculating box which is movable can easily be constructed as shown in the figure on p. 168. The frame is made of wood and it has a sloping glass window in front, and two apertures whereby the hands and arms can be inserted to carry out the necessary manipulation of the cultures. A convenient size is 3 ft. wide, 2 ft. deep and 3 ft. high.

SEPARATION OF MIXED CULTURES

1. By Plating.—The term "plating" is generally applied to the inoculation of medium in Petri dishes, usually by successive strokes or spreading. The platinum loop is charged with the bacterial mixture, pus, fragment of tissue, etc., and several strokes in

series are made on the surface of the medium in a Petri dish without recharging the wire (*vide* p. 437). When a plate is not available separate colonies may be obtained by making successive strokes on one or two slopes in tubes or screw-capped bottles.


VENTILATOR
DOOR
HINGED DOOR
HINGED FRONT WHICH CAN BE OPENED
APERTURES (FOR INTRODUCING THE ARMS) COVERED BY CALICO FLAPS.

An alternative method for Petri dishes is to employ a spreader. This is made by bending a piece of glass rod, 3 mm. diameter, at a right angle in the blowpipe flame, the short limb, used for spreading, being 1 in.

long. A small amount of the bacterial mixture is placed on the plate with the inoculating loop or capillary pipette. By means of the spreader, previously sterilised by boiling and then cooled, the material is evenly distributed over the surface. The spreader is then transferred to a second plate, which is similarly inoculated. Thus the medium in the second dish is inoculated merely with the organisms carried over by the spreader from the first.

By these methods the bacteria are gradually wiped off the wire or spreader so that they are ultimately deposited singly. Generally from each bacterium an isolated colony will grow; a single colony may be subcultured on fresh media and so yield a pure growth. *In order to ensure separation, the surface of the medium must be dry.*

2. BY PLATING DECIMAL DILUTIONS OF THE INOCULUM.—A series of tubes or bottles of melted agar or gelatin are inoculated with successive decimal dilutions of the infected material and then the medium in each tube is poured into a Petri dish and allowed to solidify. By dilution, the bacteria are separated from one another, and on incubation the resulting colonies are distributed singly throughout the solid media. (This method is also used when the number of viable organisms present in a fluid has to be ascertained, as in water and milk examinations, and in blood in cases of septicaemia.)

3. BY HEATING AND SUBSEQUENT PLATING.—This method is employed where the organisms to be obtained in pure culture are more resistant to heat than the remainder of the bacteria present. This method applies especially to spore-bearing organisms, such as the anaerobes (p. 509), the spores of which survive the heating. The mixture of bacteria is heated to 65° C. for half-an-hour and then plated. The spores form individual colonies, which may then be "picked off."

Plate cultures should have the nature of the material, and also the date, written on the glass of the Petri dish by means of a grease pencil. Agar plates are incubated in the inverted position—*i.e.* the lid of the plate is underneath and the grease-pencil writing should be on the portion of the dish containing the medium. On the other hand, gelatin, because it is liquefied by many organisms, is incubated with the lid uppermost on which the necessary pencil notes are made.

Care must be taken in *picking off single colonies*, particularly when they are very close to one another. that the point of the wire does not touch any of the neighbouring colonies. The culture should first be looked at through the medium by holding it up to the light. The lid should be removed and the dish held round the side by the thumb and middle finger of the left hand. The colonies selected should be marked by grease-pencil rings on the bottom of the dish. To pick off the colony, first sit down with both elbows on the bench. Hold the plate vertically with the left hand, then grasp the holder of the wire like a pen, with the fingers quite close to the wire. Steady the right hand by placing the little finger on the left thumb in the way artists support the hand when painting. The selected colony is then easily removed without touching the others. Lay the plate on the bench, withdraw the right hand to the other end of the holder and inoculate the required medium in the manner previously described.

Plate Culture Microscope.—Several makers produce low-power binocular magnifiers which are extremely useful for examining plate cultures of organisms; they have a long working distance so that a colony can be "picked off" the plate while using the instrument. When dealing with bacteria forming small delicate colonies, or where the colonies of the desired organism are few in number, the low-power binocular is invaluable. A magnification of ten diameters is useful for general work, but by interchangeable eye-pieces and objectives, magnifications from three to thirty diameters are available.

4. By Shake Culture in Tubes (*vide* p. 163). This method is sometimes used in the separation of anaerobic organisms.

5. By the Use of Selective Media.—Such media as desoxycholate citrate agar for the coli-typhoid-dysentery group, Dieudonné's for the cholera vibrio, the tellurite media for *B. diphtheriae*, etc., have been devised so that the majority of the organisms other than those for which the media are used will not grow, and the isolation of pure cultures is thus facilitated.

6. By Animal Inoculation.—Advantage is taken of the fact that laboratory animals are highly susceptible to certain organisms—for example, the mouse to the pneumococcus. If a mixture of organisms containing the pneumococcus—*e.g.* sputum—be inoculated subcutaneously into a mouse, the animal dies of pneumococcal septicaemia in twenty-four to thirty-six hours, and from the heart blood the organism can be obtained in pure culture. Similarly the tubercle bacillus can be isolated from contaminating organisms by inoculation of a guinea-pig. The tubercle bacillus is found in pure culture in the resulting lesions.

Other Methods.—*Vide* pp. 373, 401, 530 and 573.

INCUBATION

Students and others commencing work in a laboratory should familiarise themselves with the mechanism of the incubator, whereby any desired temperature may be constantly maintained. Incubators may be heated by electricity, gas or oil, according to the facilities of the laboratory.

All bacteriological laboratories have one or more incubators working at 37° C.. This temperature, which is the optimum for practically all pathogenic organisms, is that referred to when speaking of incubation without mentioning the temperature.

Some laboratories have a warm room heated by gas or electricity, and kept at 37° C., in which large quantities of material can be incubated. The warm room should have double doors with a space between them to act as an air-lock. To enter the room the outer door is opened, the air-lock entered, and the outer door closed. The second or inner door which leads into the room is now opened, the hot room entered, and the inner door closed. This method of entering prevents cold air from the outside lowering the temperature. When leaving the hot room the inner door is closed before the outer door is opened. The hot room has a regulating mechanism similar to the ordinary incubator to keep the temperature constant, and if electrically heated it should be fitted with a device to cut off the current for the room at the main switch, if the temperature rises above 40° C..

Other temperatures for incubation are 30° C., used for cultivating leptospirae, and 22° C. (" cool incubator "), used for certain fungi and for gelatin cultures. (Gelatin medium melts at about 24° C.)

In order to prevent drying of the medium when prolonged incubation is necessary, as in the cultivation of the tubercle bacillus, the mouths of the culture tubes are sealed with paraffin wax, or covered with special rubber caps or " hermedisks " (p. 134). Under these circumstances, however, we strongly advise that screw-capped bottles (p. 161) should be used instead of test-tubes.

METHODS OF ANAEROBIC CULTURE

(See also **Appendix**)

Obligate anaerobes are defined as organisms that will grow only in the absence of free oxygen (*vide* p. 18). It has been shown that it is not oxygen by itself which is inimical to the growth of these organisms, but that when molecular oxygen is present, a peroxide is formed, probably hydrogen peroxide, which prevents their multiplication. Anaerobes may be cultivated, therefore, either by preventing the admission of oxygen to cultures, or by destroying the peroxide as it is formed, by means of catalase derived from fresh animal or vegetable tissue.

In the *Smith-Noguchi* method, for example, a combination of these methods is used. The cultures are sealed from the air by a vaseline plug; and any peroxide that may be formed is at once destroyed by the catalase present in a piece of fresh sterile rabbit kidney. This method is described on p. 150.

The method usually employed to establish anaerobic conditions is to remove the oxygen from the atmosphere surrounding the culture, the oxygen being sometimes replaced by an inert gas.

The simplest method of securing anaerobiosis is by growing the organisms in solid media. Deep agar tubes are convenient and efficient for the purpose. The addition of 1 per cent. glucose to the medium is of value, particularly when cultivating the saccharolytic group of anaerobes. Glucose acts as a reducing agent, and further serves as a suitable pabulum for bacterial growth. The agar may be inoculated when solid by means of a long straight wire (*vide* p. 164). The colonies develop best in the depth of the tube, becoming fewer and smaller towards the surface. No growth is usually noted in the top half-inch of the medium. An alternative method is to melt the agar, cool it to 50° C. and introduce the inoculum by means of a capillary pipette. The contents of the tube are mixed by rotation between the palms of the hands. The agar is then rapidly solidified by placing the tube in cold water. The colonies develop in the deep portions of the tube, usually separated from one another.

Glucose-broth can easily be rendered completely anaerobic. Long tubes, 8 × ½ in. (Noguchi tubes), are half filled with the medium and are placed in the steamer for half-an-hour or in boiling water for five minutes. Sterile melted vaseline is then poured on the surface of the medium and the tubes are rapidly cooled. The heating removes all oxygen, and the vaseline effectively seals the medium from the air.

Inoculation is made by means of a capillary pipette after melting the vaseline. Gas-producing anaerobes should not be cultivated in this medium, as the gas formed will force out the vaseline seal.

Robertson's cooked-meat medium (p. 153) is also very useful for anaerobic work. The sterilised tissue contains reducing substances, which are effective in maintaining anaerobic conditions at the bottom of the tube. The reducing activity of the meat is shown by the pink colour in the lower layers due to the reduction of haematin.

For media in screw-capped bottles, the following method is very simple. Remove the screw-cap and replace with a cap which has been perforated and fitted with a rubber washer as for blood-culture bottles (p. 185). (A supply of these caps individually wrapped in kraft paper and sterilised can always be kept available.) Connect a fine hypodermic needle by means of ·pressure tubing to the Geryk or other vacuum pump. Pass the needle through the perforated cap and washer and commence suction. After the air has been removed, and while *suction is still proceeding*, withdraw the needle from the bottle. The rubber will close and maintain the vacuum. If gas is produced by the anaerobe it will replace the vacuum.

REMOVAL OF OXYGEN BY GROWTH OF ANOTHER ORGANISM

The growth of another organism, e.g. *B. pyocyaneus*, inside the same container will remove oxygen and permit anaerobes to grow. A convenient method is to select two Petri dishes with the bottom half of each the same size. Into one dish is poured ordinary nutrient agar, and into the other the requisite medium for growth of the anaerobe. When ready for use the agar plate is inoculated with *B. pyocyaneus*. The other plate is spread with the anaerobe and inverted over the agar plate so that the edges are in register. A broad rubber band is then slipped over the junction of the two dishes.

REMOVAL OF OXYGEN BY MEANS OF PYROGALLIC ACID AND CAUSTIC SODA

When pyrogallic acid and caustic soda are mixed together, the mixture rapidly absorbs oxygen and becomes dark brown in colour. Many types of apparatus have been devised for the cultivation of anaerobes on plates, in which this method is used for absorbing oxygen. They are, however, inconvenient, and are not specially recommended.

For media in ordinary test-tubes, Buchner's Method is applicable. The test-tube containing the medium is placed in a Buchner's tube, a stout-walled tube $8\frac{1}{2} \times 1$ in., with the lower end constricted so that the test-tube placed therein does not reach to the bottom of the tube. The tube is furnished with a well-fitting rubber bung. Some solid pyrogallic acid is placed in the bottom of the tube, strong caustic soda is added, the inoculated tube is quickly introduced, and the rubber bung immediately inserted. The oxygen is rapidly absorbed and fairly satisfactory anaerobic conditions are obtained.

McINTOSH AND FILDES' JAR

This apparatus is easy to manipulate, and the degree of anaerobiosis is easily observed by means of a methylene-blue indicator inside the jar.

The principle of the apparatus is that spongy palladium or spongy platinum acting as a catalytic agent causes the slow combination of hydrogen and oxygen to form water. The jar itself (8 × 5 in.) is made of stout glass or of metal, and has a tight-fitting lid that can be clamped down. The lid is furnished with two tubes and taps, so that hydrogen may be introduced into the jar. Suspended from the lid by means of two wires, which are connected to terminals, is a small glass or porcelain spool around which is the spongy palladium. This spongy palladium is made by immersing asbestos in a solution of palladium chloride and allowing it to dry ; on heating in the blowpipe, the palladium is deposited in a black amorphous spongy layer on the asbestos. A fine coil of resistance wire is wound round the palladinised asbestos and the ends are connected to the two wires supporting the spool, so that an electric current can be passed through and the spongy palladium heated. The spool is surrounded by wire gauze, which, on the principle of the Davy lamp, prevents an explosion of the hydrogen and oxygen mixture. The amount of current passed through the coil depends on the thickness of the resistance wire and on the voltage of the electric supply. The current passed by three carbon filament lamps in parallel (two 16 c.p., and one 8 c.p.) is suitable with the ordinary domestic supply.

Petri dishes or tubes are placed inside the jar, and also an indicator to show that anaerobiosis is maintained. This consists of a mixture in a test-tube of equal volumes of (*a*) N/10 NaOH 6 c.c., water to 100 c.c., (*b*) 3 c.c. ½ per cent. watery methylene blue, water to 100 c.c., (*c*) glucose 6 grams, water to 100 c.c., and a small crystal of thymol; the mixture is boiled until it becomes colourless, and is at once placed in the jar. This indicator, when in the jar, should remain colourless except for a slight tinge of blue at the top, which slowly disappears during the passing of the current.

The lid is clamped down and the jar connected to a hydrogen supply. The current is turned on so that the palladinised asbestos may be heated. The combination of oxygen and hydrogen takes place quietly in the jar. Water is formed, and more hydrogen enters to take the place of the oxygen consumed. After about twenty minutes all the oxygen is used up, and the tap is then turned off and the hydrogen supply disconnected. The jar is placed in the incubator, and the indicator tube containing the methylene blue should remain colourless, showing that complete anaerobiosis is established.

In spite of the wire gauze round the palladinised asbestos, explosions sometimes occur, and it is advisable always to place the anaerobic jar, if made of glass, inside a box while the current is passing through the spool. See also **Appendix.**

HYDROGEN SUPPLY

A hydrogen supply may be obtained from a Kipp's apparatus *by the action of sulphuric acid on zinc.* The gas must be purified by passing through three wash-bottles: (1) containing a 10 per cent. solution of lead acetate, to remove sulphuretted hydrogen; (2) containing a 10 per cent. solution of silver nitrate, to absorb arseniuretted hydrogen; and (3) containing a mixture of pyrogallic acid and caustic soda, to remove oxygen.

It is more convenient, however, to obtain hydrogen from a *cylinder containing the compressed gas.* The commercial hydrogen so obtained is suitable for use in the various anaerobic apparatus employed. The hydrogen cylinder cannot be connected directly to the McIntosh and Fildes' jar, as the pressure is too great. It should be fitted with a reducing valve to deliver hydrogen at a constant pressure (*e.g.* 2–3 lbs. per square inch) which can be predetermined or altered at will. The gas is then passed through a small wash-bottle containing water in order that its rate of flow may be observed and to detect when no further hydrogen is drawn into the anaerobic jar—a state which is reached when all the oxygen in the jar has combined with hydrogen.

A very simple alternative method is to attach an ordinary football bladder to the hydrogen cylinder. The gas is turned on and the bladder inflated. The gas is then turned off, the tube of the bladder closed by a screw clamp, and removed from the cylinder. The inflated bladder is connected by its tube direct to the anaerobic jar.

A considerable saving in both time and hydrogen is effected if the bulk of the air is removed from the jar, by evacuation with a water-pump or other suitable means, before passing in hydrogen.

Anaerobiosis in a closed jar may be secured *by generating hydrogen from chromium and sulphuric acid.*[1] This is a convenient improvisation if other apparatus is not available. A desiccator with a stopcock is used. The cultures are placed in the desiccator along with a dish containing chromium metal powder, to which is added 15 per cent. sulphuric acid. The stopcock is left open while the vigorous evolution of hydrogen continues, and then closed. The jar is placed in the incubator.

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[1] Rosenthal, L., *J. Bacteriol.*, 1937, **34,** 317.

CULTIVATION IN AN ATMOSPHERE WITH ADDED CARBON DIOXIDE

It has been found that certain organisms will grow only when carbon dioxide is added to the atmosphere surrounding them, e.g. *B. abortus*, and that some grow better in such atmospheres than in ordinary air, *e.g.* pneumococcus, etc.. It is also recommended that the solid CCY media (p. 109) should be incubated in an atmosphere of about 5 per cent. carbon dioxide. A convenient method [1] is to use tin containers, size 8 × 10 in., with press-down lids, and capacity of about 3½ litres.[2] (Any similar tin container which will accommodate Petri dishes may be used.) The carbon dioxide is generated in the tin itself from marble and hydrochloric acid. The cultures, either in Petri dishes (enclosed in a simple wire basket) or in tubes, are placed in the tin together with an open tube 8 × 1 in. containing 8 c.c. (excess) of 25 per cent. hydrochloric acid. A marble chip of about 0·7 gram (weight need only be approximate) is dropped into the acid and the lid pressed down. The slight increased pressure of the carbon dioxide is of no consequence. If the cultures are carefully removed and fresh ones added immediately there is no need to renew the marble and acid.

In using such closed containers there is a tendency for moisture to collect on the lid of the Petri dish. The same occurs in anaerobic jars (*vide supra*). It is recommended, therefore, before incubating to place in the lid of the dish a square piece of filter or blotting paper of such a size that it is just held in position by its four corners, *e.g.* for the usual 4 in. place a 3-in. square. The paper should not fill the top of the dish as it would, when wet, act as a seal and prevent the access of carbon dioxide to the inside of the dish.

For larger proportions of carbon dioxide an anaerobic jar may be used. Air is withdrawn by means of a filter pump and replaced by carbon dioxide from a Kipp's apparatus or cylinder, as described above for hydrogen.

OXIDATION-REDUCTION POTENTIALS

Sour milk, bacterial cultures, etc., decolorise methylene blue owing to the development of reducing conditions during bacterial growth. In order to follow up qualitative observations of these reducing effects a quantitative measure for evaluating oxidation-reduction conditions is required, and oxidation-reduction potentials enable this to be done. The principle of the method depends on the fact that when an "unattackable" electrode is immersed in a solution, an electrical potential difference is set up between the electrode and the solution, and the magnitude of this potential depends on the state of oxidation or reduction of the solution. This electrode potential (or, more shortly E_h) can be measured in millivolts and the more oxidised a system, the higher (or more positive) is the potential; in more reduced systems the potential is lower (or more negative). By measuring the electrode potential it is possible to determine and follow the reducing conditions in cultures at different periods and to grade different systems in order according to their state of oxidation or reduction. It should be borne in mind that measurements of the electrode potential of a system, *e.g.* of a bacterial culture, indicate the oxidation-reduction *intensity* of the system itself, and not its *capacity* to oxidise or reduce some other component or system.

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[1] See Gladstone and Fildes, footnote, p. 107.

[2] Obtainable from A. Gallenkamp and Co., Ltd., London.

The electrode potential of a bacterial culture may be measured accurately by electrical methods, but an approximate idea of the state of reduction may sometimes be obtained by adding various special dyes (oxidation-reduction indicators) and observing by the colour changes how much they are reduced. Such changes are in intensity of colour, not changes from one colour to another, as is the case with the indicators used for the measurement of pH. It is found that the state of oxidation or reduction of any particular dye depends on the electrode potential, so that at any given pH value, if we know the electrode potential of the solution, we can calculate the degree of reduction of the dye. Conversely, and this is more important practically, if the percentage reduction of the dye has been observed colorimetrically the corresponding electrode potential can be deduced. Different dyes are reduced over different ranges of potential; for instance, methylene blue at pH 7 is 95 per cent. in the oxidised condition at $E_h + 50$ mv., and 99 per cent. reduced at $E_h - 50$ mv., whilst neutral red is still 87 per cent. oxidised at -300 mv., and 87 per cent. reduced at -350 mv. Theoretically it should be possible by suitable choice of indicators to measure any range of E_h, but in practice experimental difficulties arise due to poising,[1] catalytic effects and the toxicity of the dyes used towards bacteria, etc.. Colorimetric E_h determinations do not reach the degree of accuracy and convenience attained in the case of pH indicators.

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[1] Corresponds to the buffering effect in pH estimation.

A few examples will suffice to illustrate the results obtained when the electrode potentials of growing bacterial cultures are measured. In a culture of *B. diphtheriae* it was found that the initial E_h of the medium, about +300 mv., fell gradually and reached −200 mv. after some forty-eight hours' incubation and the potential remained at this low level for some considerable time. With haemolytic streptococci, on the other hand, the potential fell from +300 mv. to −150 mv. in twelve hours but then rose fairly rapidly, probably owing to the formation of hydrogen peroxide. In a glucose broth culture of *B. coli*, in which gas formation occurred, the potential fell extremely rapidly, reaching −370 mv. after about one hour's incubation. The behaviour of staphylococci is roughly similar to that of *B. diphtheriae*, whilst pneumococci behave similarly to haemolytic streptococci.

Strict anaerobes are unable to proliferate in ordinary aerobic culture media unless the E_h is lowered to some extent. This lowering of the E_h, or establishment of reducing conditions may be effected in a variety of ways, such as removal of oxygen in an anaerobic jar or by means of a pyrogallol seal, or reduction may be effected by adding a reducing substance such as cysteine. It must not be assumed, however, that there is a strict line of differentiation between aerobes and anaerobes. Every grade of behaviour may be observed from the aerobic organisms, such as *M. lysodeikticus* which proliferates actively only when the oxygen supply is abundant, to the strict anaerobes which appear to require absolute exclusion of air at least in the initial stages of growth. Some organisms such as *B. coli* which are well supplied with enzyme systems of many kinds are able to multiply over a very wide range of cultural conditions. Bound up with this question is that of accessory growth substances which must be supplied to some organisms whilst others are able to synthesise their own, but this need not be discussed further here.

Oxidation-reduction potentials and oxidation-reduction indicators are employed in the testing of sewage and sewage effluents, in connection with cheese-making and the keeping qualities of beer, in the determination of vitamin C, etc.. The metabolic activities of bacteria and other cells and tissues and the functioning of enzymes are followed by observing the reduction of methylene blue in Thunberg tubes. A commonly used application of this technique is in the grading of milk and testing the quality and keeping powers of milk samples. The milk samples are incubated under standard conditions with methylene blue and the time of reduction is noted. Heavily contaminated milks show a rapid decolorisation, whilst with good quality milk there is a long lag period and reduction is slow (*vide* p. 308).

For full details of this important subject the following monograph should be consulted: *Oxidation-Reduction Potentials in Bacteriology and Biochemistry*, by L. F. Hewitt, 4th edition, 1937; published by the London County Council; obtainable from King & Son, London.

PRESERVATION OF CULTURES BY DRYING *IN VACUO*

When cultures are dried rapidly *in vacuo* they may retain their viability for many years and this is a convenient means of keeping stock strains of many organisms. For example, pneumococci and streptococci grown in blood-broth may be preserved in this way. A simple method is to dry the culture in a desiccator over calcium chloride or phosphorus pentoxide, as described for the preservation of complement on p. 267. Three drops of well-grown culture in fluid medium are placed in a wide-necked 1 c.c. ampoule and treated in the same way as the complement-containing serum. In our experience cultures of streptococci will survive for two years by this method.

A more elaborate method, which gives better results, requires the use of an exhaust pump giving a high degree of vacuum, while the water vapour given off during the drying of the culture is trapped by freezing with solid carbon dioxide ("Drikold" or "Dry Ice"). The apparatus[1] consists of a wide-bore glass tube, about 25 mm. external diameter, sealed at one end and with several lateral tubes 12 mm. external diameter. These lateral tubes are fitted with rubber pressure-tubing and strong screw clamps, to which the tubes of material to be dried are attached. To the end of the wide tube is fitted by means of a ground glass joint (or preferably flexible metal tubing) a tube of similar width having two side-arms connected to glass vessels of about 250 c.c. capacity which can be cooled by immersion in solid carbon dioxide dissolved in acetone and contained in conveniently sized commercial vacuum flasks. This tube is connected to a Hyvac pump suitably protected from moisture by tubes of phosphorus pentoxide or calcium chloride and the apparatus is exhausted. The cultures are dried in Pyrex glass tubes which are sealed *in situ* while still exhausted. These are prepared as follows: a piece of 9 mm. Pyrex tubing, 14 mm. long, has a small bulb blown at one end and a slight constriction made (for ease of sealing) 7 mm.

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[1] Obtainable from J. C. Cowlishaw, University Works, 42 Bridge Street, Manchester.

from the other end, the tube thus forming an ampoule ; it is plugged with cotton-wool and sterilised in the hot-air oven. Three drops of culture are added and the tube is then attached to one of the lateral arms of the apparatus. Similar tubes with cultures added are connected to the other lateral arms. The pump is started and the apparatus exhausted until a sufficiently high degree of vacuum is obtained. The cultures immediately froth and dry almost at once. Then they are left for about ten minutes. The screw clamps are closed and each tube containing culture is sealed *in situ* while still under vacuum by means of a small flame from a blowpipe. After sealing, each tube of dried culture is tested for vacuum (to ensure that there are no leaks) by means of a high-frequency vacuum tester connected to the A.C. mains. If a satisfactory vacuum is present a bluish-violet fluorescence is seen in the tube. As the apparatus takes some time to exhaust thoroughly and the solid carbon dioxide has to be obtained usually direct from the makers on each occasion, it is convenient to deal with a number of cultures simultaneously and the apparatus described is adapted for this purpose. See also **Appendix**—Rayner's method.

BLOOD CULTURE

In most bacterial infections of the blood in the human subject the organisms are not numerous and it is essential for their demonstration in cultures that a relatively large amount of blood, *e.g.* 5–10 c.c., should be used as the inoculum. When such quantity of blood is added to a culture medium, its natural bactericidal or bacteriostatic action may readily interfere with growth and it is therefore essential that this effect should be annulled by diluting the blood with medium. Alternatively, the antibacterial effect may be prevented by some substance incorporated in the medium; *e.g.* trypsin. While it is not necessary that the blood should remain unclotted in the medium, some workers prefer to add sodium citrate, ammonium oxalate or other anticoagulant.

Requisites :

(1) A 10 c.c. Record syringe or an "all-glass" syringe (with a firmly fitting needle) sterilised by

boiling in water for fifteen minutes; the syringe must not come into contact with any antiseptic; it should not be removed from the steriliser until it is immediately required, and the parts should be taken out of the steriliser and fitted together with the aid of forceps so that the needle, nozzle and piston are not touched by the fingers. Alternatively the syringe, if "all-glass," may be sterilised beforehand in the hot-air oven as described on p. 232, but this procedure is not recommended for the Record type, as the cement or solder is apt to melt.

(2) Gauze or cotton-wool, bandage, antiseptic (*e.g.* 4 per cent. iodine in spirit), methylated spirit, collodion, dissecting forceps, Bunsen burner or spirit lamp.

(3) 100 c.c. sterile digest broth (*e.g.* Hartley's, p. 101) in a stoppered flask, or preferably a special blood-culture bottle, described later. (0·2 per cent. sodium citrate or 1·0 per cent. ammonium oxalate may be incorporated in the medium.)

The blood is drawn by vein puncture. The skin of the patient's arm at the bend of the elbow is *thoroughly sterilised* by first washing with soap and water, then applying spirit and finally treating with the iodine solution. This is particularly necessary to obviate contamination of the culture with skin organisms—e.g. *Staphylococcus albus*. Several turns of a bandage are applied round the upper arm about the middle of the biceps to render the veins turgid, or a piece of rubber tubing firmly, but not too tightly, wound once round the arm and clipped with pressure forceps provides a convenient and easily released tourniquet for the purpose. The turgescence of the veins can be increased by the patient's alternately opening and clenching the hand. The needle of the syringe is inserted into a prominent vein and 5–10 c.c. of blood are drawn into the syringe. The tourniquet is then released. The needle is now withdrawn from the vein and detached from the syringe by means of forceps so that the nozzle is not touched by the fingers. The flask of broth is unstoppered and the mouth of the flask flamed. The blood is added to the broth and the flask re-stoppered. The blood and broth are thoroughly mixed by rotation of the flask. These operations are all done at the bedside. A spirit lamp may be used for flaming. The flask is incubated at 37° C..

The patient should raise the arm after blood has been withdrawn and firm pressure should be applied to the site of the puncture to obviate haematoma formation.

The syringe and needle should be washed out at once with cold water. The puncture wound is dressed with gauze or cotton-wool, and collodion.

When the flask has to be transported some distance to the laboratory it is advisable to stopper it with a rubber bung which has been sterilised by boiling, and inserted into the flask with flamed forceps.

In suspected cases of *enteric fever*, instead of broth as the culture medium, sterilised ox bile or 0·5 per cent. sodium taurocholate broth may be used. 5 c.c. of blood are added to 10 c.c. of bile or to 50 c.c. of taurocholate solution.

It has been pointed out by Penfold, Goldman and Fairbrother (*Lancet*, 1940, i, 65) that to obtain the best general results a range of media should be used. A medium containing saponin is of special value in isolating *Streptococcus viridans* ; broth containing glucose and trypsin serves well for staphylococci. Cooked meat medium (p. 154) is applicable for cultivating anaerobes and microaerophilic organisms from blood.

Saponin broth.—Broth with 0·2 per cent. sodium citrate and 0·1 per cent. white saponin (B.D.H.), the medium being sterilised by intermittent steaming. Blood is added in the proportion of 1 to 5 of the medium.

Glucose trypsin broth.—Broth with 1 per cent. glucose, and 1 part of filtered Liquor trypsini (Allen & Hanbury) to 10 parts of the medium.

See **Appendix**—addition of *p*-aminobenzoic acid to media.

Blood-culture Bottle.—Instead of using a flask of broth with a cotton-wool or rubber stopper, as described above, the following container is much more simple and convenient, especially when the patient is some distance from the laboratory.

It consists of a 6 oz. bottle, with a screw-cap, as used for storing nutrient agar, etc., in 100 c.c. amounts (see p. 97). A hole is punched out of the cap and the rubber washer re-inserted. In order to protect the surface of the cap and the exposed portion of the rubber washer from contamination before use, the cap and neck of the bottle are covered by a "viskap",[1] such as is used for perfume bottles. This is a cellulose preparation which is slipped on moist and allowed to dry. In so doing the viskap shrinks, moulding itself tightly to the cap and neck of the bottle. A metal slitter may be fitted under the viskap to facilitate its removal.

The apparatus is fitted up as follows:—

The bottles are supplied in a carton already washed, cleaned and capped, so that no further preparation is required. The rubber washer is removed, a $\frac{5}{16}$ in. hole punched out of the centre of the cap by means of a hollow punch, and the rubber washer re-inserted.

The medium in the bottle is a matter of choice, and the following range is useful. The different types are recognised by the colour of the viskap and the glass bead in the bottle. (1) Plain broth, white cap and bead; (2) broth + 0·1 per cent. glucose, green cap and bead; (3) broth + 0·2 per cent. sodium citrate, purple cap and bead; (4) broth + 0·5 per cent. bile salt, yellow cap and bead. The size of the viskap is No. 2 semi-opaque cut $1\frac{3}{8}$ in. The top of the viskap is coloured with cellulose paint, *e.g.* "Luc." The bead is to identify the medium on its return to the laboratory.

100 c.c. of the medium are placed in the bottle, the appropriately coloured bead added, and the perforated cap with rubber washer firmly screwed on. The bottle is now sterilised in the autoclave for fifteen minutes at 5 lbs. pressure. When the bottle is cool a metal wire slitter (sterilised in alcohol) may be bent into position over the cap and the viskap is at once slipped on. The viskaps dry in a few hours and mould themselves to the cap and neck of the bottle. The broth can be stored without deterioration. For streptococci or pneumococci the glucose broth will be found the most useful. The bile-salt broth is intended for cases of suspected enteric fever.

For use, the bottle is taken to the bedside of the patient. Just before the vein is punctured the viskap is removed by pulling up the end of the slitter. Blood (5–10 c.c.) is now withdrawn from the vein, with the usual precautions, and immediately afterwards the needle is passed through the rubber washer and the blood is expelled into the medium. The needle is withdrawn and the puncture in the washer seals itself. The bottle is shaken to mix blood and broth, and sent to the laboratory for incubation. It is advisable to wipe the exposed portion of the washer with a little antiseptic (*e.g.* spirit), in order to remove any infective material at the site of the puncture.

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[1] Made by the Viscose Development Co., Ltd., Woldham Road, Bromley, Kent.

When the culture is to be examined, after incubation, the screw-cap is removed in the ordinary way.

In order to economise medium and facilitate storage and transport, 2 oz. medical flat bottles with 35 c.c. broth may be used instead of the larger quantity. It is found that two varieties, glucose-broth and bile-salt-broth, as described above, fulfil most needs. Instead of beads for identification, a small dab of coloured paint (green for glucose, yellow for bile salt) is placed on the bottom of the bottle. In place of a viskap a piece of coloured cellulose tape (green or yellow) ½ in. wide stretched from one side of the neck of the bottle over the perforated cap to the other side is equally effective. The tape itself has a definite antiseptic action, so that it is only necessary to remove the tape before passing the needle through the rubber washer. The tape should be replaced before the bottle is returned to the laboratory. 5 c.c. of blood is a convenient amount to add.

"*Clot*" *Culture.*—When blood samples from suspected enteric fever have been submitted for the Widal test (*vide* p. 249) it is useful as a routine to cultivate the clot after the serum has been removed. If blood is taken in the early stages of the disease the Widal reaction may be negative, but blood culture will probably be positive. Moreover, enteric bacilli may be present in the blood stream at any time throughout the illness and isolation of the causative organism is the most satisfactory form of diagnosis.

If it is known that the blood has been withdrawn with strict aseptic precautions, the clot may be placed in a wide tube (8 × 1 in.) half filled with broth, or in a wide-mouth screw-capped bottle (6 oz. honey pot) containing 80 c.c. of broth. Where, however, there is any doubt as to the presence of contaminating organisms, and this is always a possibility when specimens of blood are sent to the laboratory from a distance, the clot should be transferred directly to a tube of sterile ox bile. After incubation overnight the bile culture is examined for enteric organisms in the usual manner.

After incubation for eighteen to twenty-four hours, films are made from the blood-broth mixture and stained by Gram's method. If organisms are noted, sub-inoculations are made on a plate of agar or other suitable medium by the successive stroke method (*vide* p. 167). The subcultures are incubated and the organisms developing are identified as far as possible by their microscopic characters and colony appearances. If further investigations are required for accurate identification, single colonies are "picked off" on to slopes and the resulting cultures are studied.

Where an infection with the enteric group is suspected, it is convenient to plate directly from the blood culture on MacConkey's medium to obtain the characteristic colonies on this medium.

Even when no organisms can be detected in films from the primary blood culture, it is advisable to make sub-inoculations, as scanty organisms may not be observed, but still develop colonies in subculture.

If no result is obtained after twenty-four hours' incubation, the blood-broth should be incubated continuously for *at least* four days, films and sub-inoculations being made each day.

A convenient instrument for drawing aseptically a blood sample from a vein is the so-called *Behring Venule.*[1] It also serves as a container for transmitting the sample to the laboratory, and provides medical practitioners with a simple means of carrying out the clinical technique of blood culture. It consists of an evacuated receiving-tube closed by a hollow rubber stopper, and a needle attached to a narrow glass connecting-tube which passes through the stopper and is kept closed by a simple valve arrangement (see diagrams, p. 188). The needle is enclosed in a sealed extension of the connecting-tube. Both the needle and interior of the receiving-tube are sterile. The needle is exposed by breaking off the covering tube and is inserted into a vein; the valve is then opened by bending the "knee" formed by the receiving and the connecting tubes. Blood is automatically drawn into the receiving-tube and thereafter the valve is allowed to close. In addition to the "venule" for collecting ordinary blood samples, special

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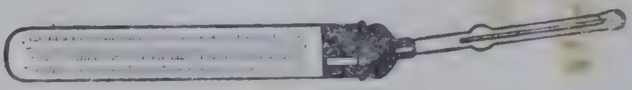
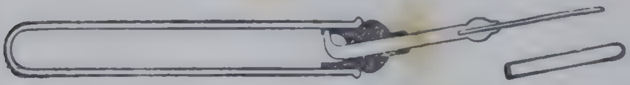
[1] These instruments can be obtained from Bayer Products Ltd., London

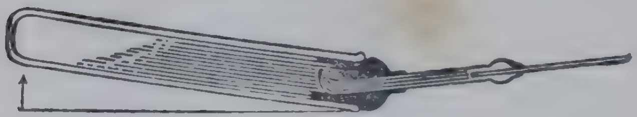
"venules" are obtainable—*e.g.* containing broth for blood culture, bile for blood culture in enteric fever, glass beads to defibrinate the blood, citrate solution to obviate coagulation, etc..



Venule before use.



Sectional view—ready for use.



Sectional view—valve opened in use.

Reproduced by permission of Bayer Products Ltd., London.

THE AGAR-BLOCK METHOD OF ØRSKOV FOR STUDYING THE MORPHOLOGY OF GROWING BACTERIAL CULTURES

This method has been applied by Ørskov[1] and others for the morphological study of *Actinomyces* and it allows the maintenance of living cultures under continuous observation.

Cubes of suitable size are cut out of an agar plate by means of a sterilised knife. These cubes should not exceed 3–4 mm. in thickness. They are transferred with the knife to a sterilised microscopic slide. The agar is now inoculated with the organism by a fine stroke. With first a low-power objective the stroke can be defined and then with a higher power an area can be found where the bacteria lie suitably scattered. With a suitable lamp and objective and closing down of the diaphragm young bacteria appear as strongly refractile and well-defined bodies. The area is then registered by means of the vernier scales on the mechanical stage. The slide is removed and placed in a Petri dish with a piece of moist filter paper in the bottom and the dish is incubated at a suitable temperature. The selected area is then examined at intervals and the changing features observed. In this way the development of individual bacteria can be studied and also that of colonies at each stage. (A microscope incubator, heated by electricity, by means of which a colony can be observed microscopically throughout its period of growth is also very convenient for these and similar studies.)

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[1] Ørskov, J., *Investigations into the Morphology of the Ray Fungi*, 1923, Copenhagen.

DISPOSAL OF CULTURES

Cultures to be discarded should be killed by heat or antiseptics before the container is cleaned for re-use.

In the case of non-sporing organisms, it is sufficient to remove the cotton-wool plugs, and immerse the tubes and plugs in a large basin of 3 per cent. lysol or cresol.

When screw-capped bottles are used the cap is completely unscrewed, and both it and the bottle placed in the lysol solution.

Cultures of the tubercle bacillus and sporing organisms, such as *B. tetani*, *B. anthracis*, etc., *should be sterilised by autoclaving.*

PERSONAL PRECAUTIONS IN BACTERIOLOGICAL LABORATORY WORK

It is essential to wear an overall while at work. If any material containing pathogenic organisms drops on the bench, floor, clothes, apparatus, etc., it should be sterilised at once with 3 per cent. lysol or 1:1000 perchloride of mercury solution. If the hands become contaminated they should be sterilised in a basin of dilute lysol or perchloride of mercury solution, and workers should make it a rule always to sterilise and wash the hands after completing any bacteriological work.

It must be emphasised that in the laboratory labels must never be licked. There is always a grave risk of infection by this habit. Labels should be moistened either by a drop of water on the finger or by a pledget of wet cotton-wool.

Precautions to be taken in carrying out special methods are referred to later.

CHAPTER VI

STAINING METHODS

As bacteria consist of clear protoplasmic matter, differing but slightly in refractive index from the medium in which they are grown, it is difficult, except by special methods of illumination, to see them in the unstained condition.

Staining, therefore, is of importance, not only for the recognition of bacteria, but also in virtue of the fact that by special methods certain organisms, such as tubercle and diphtheria bacilli, may be differentiated microscopically from others.

Bacterial protoplasm reacts to stains in a manner somewhat similar to the nuclear material of tissue cells, and therefore the various basic dyes are the commonest stains employed. The action of these stains may be intensified by the use of mordants such as phenol or weak alkalis, by the application of heat, or by prolonging the time of staining. Some organisms have a greater affinity for dyes than others, so that when stained and then treated with a decolorising agent they still retain the stain while others lose it. By such means it is often possible to differentiate species of bacteria in a mixture.

METHODS OF MAKING FILM OR SMEAR PREPARATIONS

Before describing the various staining processes, details of the methods employed in making films must be considered.

Film preparations are made either on cover-slips or on the ordinary 3 × 1 in. glass slides, usually the latter. It is essential that the cover-slips or slides should be perfectly clean and free from grease, otherwise uneven films will result.

Cover-slips.—These should be $\frac{3}{4}$ or $\frac{7}{8}$ in. square, and of No. 1 thickness. (Thicker cover-slips—No. 2—may prevent the oil-immersion objective from coming near enough for the specimen to be focussed.) They are cleaned by placing them in a mixture of nitric acid, 6 parts; potassium bichromate, 6 parts; water, 100 parts. They should be dropped one by one into the fluid. The solution is contained in an evaporating dish and boiled. The cover-slips are then well washed, first in tap water and then in distilled water, and stored in a stoppered jar in 50 per cent. spirit. Before use they are dried with a soft clean cloth, such as an old handkerchief.

Slides.—These may be treated in a manner similar to cover-slips. A quicker and quite satisfactory method for ordinary routine use is to moisten the finger with water, rub it on the surface of some fine sand soap such as "Bon ami," and then smear the surface of the slide. After removing the soapy film with a clean cloth the surface is clean and free from grease. If the slide is perfectly clean a drop of water can be spread over its surface in a thin even film; otherwise the water collects into small drops and a film cannot be made.

After the films have been made and examined the slides should be discarded. They should not be cleaned and used again.[1]

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[1] In war time, difficulties of supply may necessitate slides being re-used. They should be boiled for ten minutes in 5 per cent. soft soap solution, and then *both* sides cleaned with "Bon ami" or similar soap. It is essential to remove the film of bacteria in this way, otherwise when the slide is used again, the organisms will re-stain and cause erroneous conclusions. We have seen serious errors in diagnosis following the re-use of slides not properly cleaned. It is for this reason that we have recommended above that used slides should be discarded.

In the case of fluid materials, such as broth cultures, urine, sputum, pus, etc., one loopful (or more) is taken up with the inoculating wire and is spread thinly over the slide. A little experience will soon determine the amount required, and in spreading the films it will be found that there are both thick and thin portions, which is not disadvantageous. The slide is then held in the palm of the hand high over a Bunsen flame and dried. The film is fixed either by passing the slide three times slowly through the flame, or by heating through the glass slide. In the latter method the slide is held, film upwards, in the top of the Bunsen flame for a few seconds so that the slide becomes hot. Care must be taken not to char the film, and when the slide is just too hot to be borne on the back of the hand, fixation is complete.

In making films on cover-slips and staining them, Cornet's forceps is used to hold the slip in a horizontal position, the forceps resting on the bench.

Films on cover-slips require a minimum of time for fixing owing to the thinness of the glass.

With solid material, such as cultures on agar, etc., it is necessary to place a loopful of clean water on the slide. The loop is then sterilised and a minute quantity of material, obtained by just touching the growth, is transferred to the drop, thoroughly emulsified, and the mixture is spread evenly on the slide. The resulting film is fixed and dried as above. *Beginners are very apt to take more material than necessary from the culture and thus make too thick films.*

STAINING

The method of staining varies with the nature of the preparation (film or section).

FILMS

The stains are poured directly or filtered on to the slide. When staining is completed, the dye is washed off with water, and the slide is allowed to dry in the vertical position or is placed between two sheets of white fluffless blotting-paper or filter paper. The drying of the film is completed over the Bunsen flame. Such stained films may be mounted in Canada balsam under a cover-slip, or may be examined unmounted with the oil-immersion lens, a small drop of cedar-wood oil being placed directly on the film. If it is desired to mount the preparation later, the oil can be removed with xylol.[1]

TISSUE SECTIONS

The sections being embedded in paraffin (*vide* p. 230), it is necessary to remove the paraffin so that a watery stain may penetrate. The paraffin is first removed with xylol,[1] the xylol removed with alcohol,[2] and the alcohol replaced by water. The staining process is then proceeded with. After staining, the section must be dehydrated with absolute alcohol, then cleared in xylol and finally mounted in Canada balsam under a cover-slip. The Canada balsam (which is a resin) is dissolved in xylol in order to render it of suitable consistence.

Technique.—The slide bearing the paraffin section is placed in a jar of xylol for some minutes to remove the paraffin. The section is then treated with a few drops of 95 per cent. alcohol, when it immediately becomes opaque. A few drops of 50 per cent. spirit are poured on, and the slide is finally washed gently in water. If the tissue has been fixed in any mercuric chloride preparation, such as Zenker's fluid, the section should be treated with Gram's iodine solution for a few minutes (*vide* p. 200), then with spirit and finally water. The sections are now ready to be stained. After staining and washing with water, the slide is wiped all round the section with a clean duster to remove excess of water. The bulk of the water in the section may be removed by pressing between fluffless blotting-paper. The section is *immediately* treated with a few drops of spirit, then absolute alcohol. The slide is again wiped all round the section, a few more drops of absolute alcohol are poured on, and the slide is then immersed in xylol. When cleared, the slide is removed, and excess of xylol round the section is wiped away, a drop of Canada balsam is applied and the section mounted under a No. 1 cover-slip. It is essential that the section should not be allowed to dry at any period of the process, and that dehydration with absolute alcohol should be complete in order that the section may be thoroughly cleared.

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[1] Benzol may be substituted for xylol.

[2] Industrial methylated spirit (not mineralised) may be used for making up stains, decolorising, dehydration of tissues and treatment of sections instead of rectified spirit. The type known as "Toilet spirit, acetone free (66 O.P.)," is quite satisfactory. Similarly, industrial methylated spirit, absolute, 74 O.P. can be used instead of absolute alcohol for staining, dehydration and histological technique. Not only are these industrial spirits much cheaper than rectified spirit and absolute alcohol, but permits for obtaining them duty-free are more readily granted by the Customs Authorities.

When the bacteria are readily decolorised by alcohol, aniline-xylol (aniline, 2 parts ; xylol, 1 part) should be used for dehydration. After washing, when the slide has been wiped round the section, the preparation is blotted and then treated with the aniline-xylol mixture, which clears as well as dehydrates. The aniline-xylol is then replaced by xylol. This can be done conveniently by holding the slide almost vertically and dropping xylol from a drop bottle on to the slide just above the section. The xylol flows over the section and quickly removes the aniline. The preparation is mounted immediately in Canada balsam.

D.P.X. MOUNTING MEDIUM

A new mounting medium which replaces Canada balsam has been devised by Kirkpatrick and Lendrum.[1] It consists of polystyrene (a synthetic resin) dissolved in xylol, with a plasticiser—dibutyl phthalate—to prevent shrinking and ensure flexibility. The mountant termed D.P.X. is made up as follows :—

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[1] *J. Path. Bact.*, 1939, **49**, 592 ; 1941, **53**, 441.

Mix dibutyl phthalate (B.D.H.) . . .	5 c.c.
with pure xylol	35 c.c.
and dissolve "Distrene 80"[1] . .	10 grams

D.P.X. medium is water-clear, inert and does not become acid or cause fading of stained preparations. It is used in the same way as Canada balsam. From experience we can recommend this mounting medium in place of Canada balsam.

SIMPLE STAINS

These show not only the presence of organisms, but also the nature of the cellular content in exudates.

METHYLENE BLUE

Of the many preparations of this dye, Löffler's methylene blue is perhaps the most useful :

Saturated solution of methylene blue in alcohol	30 c.c.
Solution of caustic potash in water (1 : 10,000)	100 c.c.

(This caustic potash solution is made by adding 1 c.c. of a 1 per cent. solution to 99 c.c. of water.)

Films.—Stain for three minutes, then wash with water. This preparation does not readily over-stain.

Sections.—Stain for five minutes or longer. The application of the alcohol during dehydration is sufficient for differentiation. Aniline-xylol can also be used for dehydration and clearing.

POLYCHROME METHYLENE BLUE

This is made by allowing Löffler's methylene blue to "ripen" slowly. The stain is kept in bottles, which are half filled and shaken at intervals to aerate thoroughly the contents. The slow oxidation of the methylene blue forms a violet compound which gives the stain its polychrome properties. The ripening takes twelve months or more to complete. The preparation is used in a manner similar to Löffler's methylene blue; it is employed in McFadyean's reaction (*vide* p. 415).

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[1] Obtainable from Messrs. Honeywill & Stein, Ltd., 21 St. James's Square, London, S.W.1.

CARBOL THIONIN

Stock Solution :

Thionin	1 gram
Phenol 1 : 40 watery solution . .	100 c.c.

For Use :

Stock solution	1 part
Distilled water	3 parts

(Filter before use.)

This stain is useful for demonstrating in tissues such organisms as typhoid and glanders bacilli.

(1) Stain sections, five to ten minutes.

(2) Wash well with water.

(3) Differentiate in a bowl of water to which a few drops of acetic acid have been added.

(4) Wash well with water.

(5) Blot, dehydrate with absolute alcohol, clear in xylol and mount in Canada balsam.

DILUTE CARBOL FUCHSIN

Made by diluting Ziehl-Neelsen's stain (p. 204) with ten to fifteen times its volume of water. Stain for ten to twenty-five seconds and wash well with water. Over-staining must be avoided, as this is an intense stain, and prolonged application colours the cell protoplasm in addition to nuclei and bacteria.

NEGATIVE STAINING

"Negative Staining" is exemplified by Burri's India ink method which was formerly used for the spirochaete of syphilis. A small quantity of India ink is mixed on a slide with the culture or other material containing bacteria, and then by means of another slide or loop a thin film is made, allowed to dry, and examined. The bacteria or spirochaetes are seen as clear transparent objects on a dark-brown background.

Fleming's Nigrosin Method

A 10 per cent. solution of nigrosin (Gurr) is made in warm distilled water (solution is effected in about an hour) and filtered. Formalin 0·5 per cent. is added as a preservative. This keeps indefinitely. A small drop of the dye is placed on a slide, bacteria are mixed with it and a smear is made either with the loop or with another slide. (A number of preparations can be made on the same slide.) Alternatively a film of bacteria is made on a slide in the ordinary manner and fixed by heat. A drop of nigrosin is placed at one edge of the slide and spread by means of another slide over the bacterial film. Dry and examine. If mounted in Canada balsam under a cover-slip, the preparation is permanent.

Nigrosin gives an absolutely homogeneous background and this is the simplest method of making a preliminary examination of a culture to show shape, size and arrangement of bacteria.

Most bacteria stand out as clear objects on a dark field, but some bacilli, such as those of the coliform and haemophilic groups, show in their central portion a slightly dark patch somewhat resembling a nucleus. This is due to the fact that in drying they develop a shallow depression in which some of the nigrosin lies.

The method is of value in the preliminary examination of the spore-bearing anaerobes. In these the spores are larger than the bacilli, so that when the nigrosin film is slightly thicker than usual the spores stand out as clear bright spaces while the bacillary bodies are slightly overlaid with the nigrosin. (See also p. 209.)

The use of nigrosin in demonstrating capsules is given on p. 210.

GRAM'S STAINING METHOD

This is one of the most important methods in bacteriology, and must be employed for the diagnostic identification of certain organisms, such as the gonococcus.

The principle of the method is as follows:—certain bacteria when treated with one of the para-rosaniline dyes such as methyl-violet or gentian-violet, and then with iodine, "fix" the stain so that subsequent treatment with a decolorising agent—*e.g.* alcohol or aniline—does not remove the colour. Other organisms, however, are decolorised by this process (*vide* p. 203). If a mixture of various organisms be thus stained and subjected to the decolorising agent, it will be found that some species retain the dye, and these are termed "Gram-positive," whereas others are completely decolorised and are designated "Gram-negative." In order to render the decolorised organisms visible, and to distinguish them from those retaining the colour, a contrast or counter-stain is then applied. This contrast stain is usually red, in order that the Gram-negative organisms may easily be differentiated from the Gram-positive organisms, which retain the original violet stain. Gram's method can be carried out only with the basic para-rosaniline dyes—*e.g.* methyl-violet, crystal-violet, gentian-violet (which is a mixture of the two preceding dyes) and victoria blue. Methyl-violet and gentian-violet are the usual stains employed, but crystal-violet is a purer stain and can be used to advantage instead of the former. The iodine solution should not be kept too long, but used shortly after being made.[1]

WEIGERT'S MODIFICATION

Solutions employed—

(1) Carbol Gentian-violet [1] :

Saturated alcoholic solution of gentian-violet	1 part
5 per cent. solution of phenol in distilled water	10 parts

(This mixture should be made up each day, as it tends to precipitate)

(2) Gram's Iodine [2] :

Iodine	1 gram
Potassium iodide	2 grams
Distilled water	300 c.c.

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[1] Alternatively 0·5 per cent. solution of crystal-violet or methyl-violet 6 B. in distilled water may be used. This keeps well and is preferable to the original formula given above.

[2] See footnote on p. 200.

(3) Aniline-xylol :	
Aniline	2 parts
Xylol	1 part
(4) Dilute Carbol Fuchsin :	
Ziehl-Neelsen's carbol fuchsin (p. 204)	1 part
Distilled water	9 parts

PROCEDURE

(a) *Films.*—The film is made, dried and fixed in the usual manner.

(1) Stain with carbol gentian-violet (two to three minutes).

(2) Pour off stain, replace with Gram's iodine solution, and allow to act for one minute.

(3) Dry thoroughly by blotting.

(4) Decolorise with aniline-xylol, using several changes until the stain ceases to be removed.

Breathing on the slide after the first application of aniline hastens decolorisation.

Now examine at this stage under the low power of the microscope ; the nuclei of the pus cells should be of a pale-violet colour ; if the nuclei are deeply stained, then decolorisation is incomplete.

(5) Wash with several changes of xylol and dry.

(6) Counter-stain with dilute carbol fuchsin, ten to twenty-five seconds. Wash with water and dry.

(b) *Sections.*—Counter-stain first with carmalum[1] for ten minutes, and then proceed as above. After (5), the sections will be cleared, and can at once be mounted in Canada balsam.

JENSEN'S MODIFICATION

This modification can be recommended particularly to those commencing staining methods.

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[1] *Carmalum :* carminic acid, 1 gram ; potassium alum, 10 grams ; distilled water, 200 c.c. ; dissolve with gentle heat ; filter and add formalin, 1 c.c., as preservative.

Solutions required—

(*a*) Methyl-violet, 6 B.,[1] 0·5 per cent. solution in distilled water.

(The solution should be made up in bulk and filtered. It keeps indefinitely, and does not precipitate, but should be filtered again before use.)

(*b*) Iodine Solution (Lugol's iodine)[2]:

Iodine	1 gram
Potassium iodide	2 grams
Distilled water	100 c.c.

Note that the iodine solution is three times stronger than the original Gram's iodine.

(*c*) Counter-stain.—Neutral Red Solution :

Neutral red	1 gram
1 per cent. acetic acid	2 c.c.
Distilled water	1000 c.c.

Film Preparations.—Smears are made, dried and fixed in the usual way.

(1) Pour on methyl-violet solution and allow to act for twenty to thirty seconds.

(2) Pour off excess of stain, and, holding the slide at an angle downwards, pour on the iodine solution so that it washes away the methyl-violet. Allow the iodine to act for a half to one minute.

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[1] Crystal-violet in the same proportion is recommended as an alternative.

[2] Iodine solution does not keep well and it is convenient, especially where stains are distributed from a central source, to have potassium iodide and iodine mixed ready for solution when required. Potassium iodide tends to be hygroscopic and must be dried, otherwise the mixture becomes sticky and lumpy. Place the potassium iodide in a thin layer in a Petri dish overnight in a desiccator over calcium chloride. Mix two parts of potassium iodide by weight with one part of iodine in a mortar. Weigh out at once amounts of 7·5 grams and place them in 1 oz. screw-capped bottles (p. 97) and screw down the caps. This is sufficient for 250 c.c. of solution. The mixture keeps indefinitely and easily " pours " from the bottle. For use place the contents of one bottle into an empty 10 oz. screw-capped bottle. Add about 50 c.c. distilled water and agitate until the iodine is dissolved. Make up to 250 c.c. with distilled water.

(3) Wash off the iodine with spirit, and treat with fresh spirit until colour ceases to come out of the preparation. This is easily seen by holding the slide against a white background.

(4) Wash with water.

(5) Apply counter-stain for thirty to sixty seconds.

(6) Wash with water and dry between blotting-paper.

This method is very simple, and gives excellent results with freedom from deposit.

Dilute carbol fuchsin (1 : 15) applied for twenty to thirty seconds may be substituted with advantage as a counter-stain for routine work, but for demonstrating the gonococcus and other intracellular Gram-negative bacteria the neutral-red counter-stain should be used.

For the gonococcus and meningococcus in films, Sandiford's counter-stain is useful, particularly when the organisms are scanty.

Malachite green	0·05 gram
Pyronine	0·15 gram
Distilled water . . . . to	100 c.c.

(The stain keeps for about a month.) Apply the counter-stain for two minutes, flood off with water (but do not wash) and blot. Cells and nuclei stain bluish green. Gram-positive organisms are purple-black and gonococci red.

KOPELOFF AND BEERMAN'S MODIFICATION [1]

The following method has been found useful for class work and is recommended for beginners :—

Films—

(1) Make a thin film, dry in the air and fix with the least amount of heat necessary to kill the organisms.

(2) Flood slide with stain made up as follows :

Stain :

1 per cent. aqueous solution of methyl-violet, 6 B.	30 parts
5 per cent. solution of sodium bicarbonate	8 parts

Allow to remain on the slide for five minutes or more.

(The above solution is apt to precipitate within a few days ; it acts quite well without the addition of the bicarbonate solution and will keep indefinitely.)

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[1] Kopeloff, N., and Beerman, P., *Proc. Soc. Exp. Biology*, 1922–1923, **20**, 71.

(3) Wash off excess of stain with iodine solution and allow to act for two minutes.

Iodine Solution :

Iodine	2 grams
Normal solution of sodium hydroxide .	10 c.c.
Distilled water.	90 c.c.

(4) Drain off the excess of iodine and add acetone (100 per cent.) drop by drop until no colour is seen in the washings.

(It should be noted that decolorisation is very rapid, requiring ten seconds or less, and the time should be reduced to a minimum.)

(5) Wash slide in water.

(6) Counter-stain for ten to thirty seconds with 0·05 per cent. aqueous solution of basic fuchsin.

(7) Wash in water, blot and dry in the air.

Sections—

(1) Remove paraffin with benzol or xylol.

(2) Treat the section with spirit and wash in water.

(3) Flood with the stain and allow to act for five minutes.

(4) Wash off excess of stain with the iodine solution and allow to act for two minutes.

(5) Decolorise with acetone (*vide supra*).

(6) Wash slide in water.

(7) Counter-stain for ten to thirty seconds.

(8) Wipe carefully around the section to remove as much water as possible, dehydrate quickly in absolute alcohol, clear in benzol or xylol and mount in Canada balsam.

If Gram's method is properly carried out, Gram-positive organisms and fibrin are stained dark violet in colour. Gram-negative organisms, the nuclei and protoplasm of pus cells and tissues cells are stained pink with the counter-stain.

To obviate errors from over-decolorising, a film of a known Gram-positive organism (*e.g.* a pure culture of *Staphylococcus aureus*) may be made at one side of the pus film. This " control spot " is stained along with the film. For the recognition of Gram-negative organisms, such as gonococci or meningococci in pus, the "control-spot" must retain the violet stain while the nuclei of the pus cells are stained only with the counter-stain.

REACTIONS OF CERTAIN ORGANISMS TO GRAM'S STAIN

Positive	*Negative*
Staphylococci	Gonococcus
Streptococci	Meningococcus
Pneumococcus	Diplococcus catarrhalis
Micrococcus tetragenus	B. melitensis and B. abortus
B. diphtheriae and group of diphtheroid bacilli	Pneumobacillus of Friedländer
Tubercle bacillus. (This requires a special method, as the organism is *not usually stained* by the ordinary Gram's method)	Koch-Weeks bacillus
Smegma bacillus	Diplo-bacillus of Morax
Leprosy bacillus	B. proteus
Actinomyces	B. pyocyaneus
B. anthracis	B. mallei
B. tetani	The coli-typhoid group, B. enteritidis of Gaertner and allied organisms, and the dysentery group of bacilli
B. welchii	Vibrios—*e.g.* V. cholerae
B. sporogenes	B. pestis
Vibrion septique	B. influenzae (Pfeiffer)
B. botulinus	B. pertussis
	B. fusiformis
	Spirochaetes

Note.—The above table refers only to young cultures, as degenerated organisms belonging to the Gram-positive class may react negatively.

STAINING OF TUBERCLE AND OTHER ACID-FAST BACILLI

ZIEHL-NEELSEN METHOD

The tubercle bacillus does not stain with the ordinary aniline dyes, apparently on account of its fatty composition which prevents penetration of the stain. The principle of staining this organism is the following.

By the use of a powerful staining solution which contains a mordant, and the application of heat, the dye can be made to penetrate the bacillus. Just as the organism is resistant to ordinary stains, it also tends to resist decolorisation. Once stained, the tubercle bacillus will withstand the action of powerful decolorising agents for a considerable time and thus still retains the stain when everything else has been decolorised.

The stain used consists of basic fuchsin, with phenol as the mordant. The dye is basic and its combination with a mineral acid produces a compound which is yellowish brown in colour and is readily dissolved out of all structures except acid-fast bacteria. Any strong acid can be used as a decolorising agent, but 20 per cent. sulphuric acid (by volume) is usually employed. See also **Appendix.**

In order to show what has been decolorised, and to form a contrast with the red-stained bacilli, the preparation is counter-stained with methylene blue.[1]

Ziehl-Neelsen's (strong) Carbol Fuchsin :

Basic fuchsin	1 gram
Absolute alcohol	10 c.c.
Solution of phenol (1 : 20)	100 c.c.

Dissolve the dye in the alcohol and add to the phenol solution.

An alternative and quicker method is :

Basic fuchsin (powder)	5 grams
Phenol (cryst.)	25 grams
Alcohol (95 per cent. or absolute)	50 c.c.
Distilled water	500 c.c.

Dissolve the fuchsin in the phenol by placing them in a one-litre flask over a boiling water-bath for about five minutes, shaking the contents from time to time. When there is complete solution add the alcohol and mix thoroughly. Then add the distilled water. Filter the mixture before use.

Films.—These are made, dried and fixed in the usual manner :

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[1] Malachite green is also recommended as a counter-stain in the Ziehl-Neelsen method. A stock solution of 1 per cent. in distilled water is made, and for use a small quantity is diluted with distilled water in a drop bottle so that fifteen to twenty seconds' application of the weak stain gives the background a pale green tint. Deep counter-staining must be avoided. The pale green background is pleasant for the eyes, and is thought by some workers to make scanty organisms more easily noticed.

(1) Flood the slide with filtered carbol fuchsin and heat until steam rises. Allow the preparation to stain for five minutes, heat being applied at intervals to keep the stain hot. The stain must not be allowed to evaporate and dry on the slide.

(2) Wash with water.

(3) Immerse the slide in 20 per cent. sulphuric acid. The red colour of the preparation is changed to yellowish brown. After about a minute in the acid remove the slide, wash with water and place it in the acid again. This process should be repeated several times. The object of the washing is to remove the compound of acid with stain and allow fresh acid to gain access to the preparation. The decolorisation is finished when, after washing, the film is a faint pink.

(4) Wash the slide well in water.

(5) Treat with 95 per cent. alcohol for two minutes.

(6) Wash with water.

(7) Counter-stain with Löffler's methylene blue or dilute malachite green for ten to thirty seconds.

(8) Wash, blot, dry and mount.

Acid-fast bacilli stain bright red, while the tissue cells and other organisms are stained blue or green according to the counter-stain used.

Other organisms are " acid-fast " in addition to the tubercle bacillus. The most important in diagnostic work is the smegma bacillus, which is frequently found in samples of urine. Treatment with spirit in addition to acid may, however, decolorise this type of organism, whereas the tubercle bacillus is both acid- and alcohol-fast. The decolorisation with spirit is therefore important when examining urine for the presence of the tubercle bacillus.

It should be noted that in films stained by Ziehl-Neelsen's method, red-stained organisms in the midst of hyaline material must not be regarded as tubercle bacilli, as such material may be resistant to decolorisation.

Sections—

(1) Sections are treated with xylol to remove paraffin, then with spirit, and finally washed in water.

(2) Stain with Ziehl-Neelsen's stain as described for films, but heat gently, otherwise the section may become detached from the slide.

(3) Wash with water.

(4) Decolorise with 20 per cent. sulphuric acid as for films. The process takes longer owing to the thickness of the section, and care must be exercised in washing, to retain the section on the slide.

(5) Wash well with water.

(6) Counter-stain with methylene blue or malachite green for a half to one minute.

(7) Wash with water.

(8) Wipe the slide dry all round the section, blot with filter paper or fluffless blotting-paper, and treat with a few drops of absolute alcohol. Pour on more absolute alcohol, wipe the slide again and immerse it in the xylol jar.

(9) Mount in Canada balsam.

Leprosy bacilli are also acid-fast, but not to the same degree. They are stained in smears or sections in the same way as the tubercle bacillus, except that 5 per cent. sulphuric acid is used for decolorisation.

STAINING OF THE DIPHTHERIA BACILLUS

The diphtheria bacillus gives its characteristic staining reactions best in a young culture (eighteen to twenty-four hours) on a serum medium (*vide* p. 126).

NEISSER'S METHOD (Modified)

The following modification of Neisser's method gives better results than the original :—

Solution A :	Methylene blue	1 gram
	Absolute alcohol . . .	50 c.c.
	Glacial acetic acid . . .	50 c.c.
	Distilled water	1000 c.c.

Solution B :	Crystal-violet	1 gram
	Absolute alcohol	10 c.c.
	Distilled water	300 c.c.

Counter-Stain :

(*a*)	Chrysoidin	1 gram
	Distilled water	300 c.c.
	(Dissolve by gentle heat, and filter)	
or (*b*)	Bismarck-brown	1 gram
	Distilled water	500 c.c.

(1) Mix together two parts of solution A and one part of solution B. Stain films in the mixture for a few seconds.

(2) Counter-stain with chrysoidin or Bismarck-brown for thirty seconds (or longer if necessary).

(3) Wash rapidly in water, blot and dry.

The volutin granules of the diphtheria bacillus appear bluish black, while the protoplasm is stained yellowish brown.

A further modification of this method gives excellent results :—

(1) Stain with Neisser's methylene blue solution A (p. 206) for three minutes.

(2) Wash off with dilute iodine solution (iodine solution of Kopeloff and Beerman's modification of Gram's method, p. 202, diluted 1 in 10 with water) and leave this solution on the slide for one minute.

(3) Wash in water.

(4) Counter-stain with neutral red solution for three minutes using the same solution as that employed in Jensen's modification of Gram's method (p. 200).

(5) Wash in water and dry.

By this method the bacilli exhibit deep blue granules, the remainder of the organism assuming a pink colour.

ALBERT'S METHOD

Laybourn's modification,[1] in which malachite green is substituted for methyl-green, is given here instead of the original method.

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[1] *J. Amer. Med. Assoc.*, 1924, **83**, 121.

Solution 1

Toluidin blue	0·15 gram
Malachite green	0·2 ,,
Glacial acetic acid	1 c.c.
Alcohol (95 per cent.)	2 c.c.
Distilled water	100 c.c.

Dissolve the dyes in the alcohol and add to the water and acetic acid.

Allow to stand for one day and then filter.

Solution 2

Iodine	2 grams
Potassium iodide	3 ,,
Distilled water	300 c.c.

Note.—The iodine solution used in Jensen's modification of Gram's method (p. 200) works equally well.

Technique—

Make film preparation and fix by heat.
Apply solution 1 for three to five minutes.
Wash in water and blot dry.
Apply solution 2 for one minute.
Wash and blot dry.

By this method the granules stain bluish black, the protoplasm green and other organisms mostly light green.

This method can be recommended for routine use.

STAINING OF SPORES

If spore-bearing organisms are stained by ordinary dyes, the body of the bacillus is deeply coloured, whereas the spore is unstained and appears as a clear area in the organism. It has been supposed that the envelope prevents the stain from penetrating the protoplasm of the spore. Once the spore is stained, it tends to retain the dye after treatment with decolorising agents, and in this respect behaves similarly to the tubercle bacillus (*q.v.*).

The following is a simple and satisfactory method for staining spores :—

Films, which must be thin, are made, dried and fixed in the usual manner with the minimum amount of heating.

(1) Stain with Ziehl-Neelsen's carbol fuchsin for three to five minutes, heating the preparation until steam rises.

(2) Wash in water.

(3) Treat with 5 per cent. sodium sulphite solution for thirty seconds.

(4) Wash with water.

(5) Counter-stain with 1 per cent. aqueous methylene blue for one minute.

(6) Wash in water, blot and dry.

It is advantageous after (2) to treat with 30 per cent. ferric chloride for a minute or two and then proceed to (3) without washing in water.

The spores are stained bright red and the protoplasm of the bacilli blue.

It should be noted that the spores of some bacteria are decolorised more readily than those of others.

Fleming's nigrosin method as described on p. 197 is a simple and effective means of demonstrating spores, which stand out as clear unstained spaces on a dark background.

FLEMING'S METHOD FOR DEMONSTRATING SPORES BY CARBOL FUCHSIN AND NIGROSIN

(1) Make films on a slide in the usual way and dry.
(2) Stain for five minutes with hot carbol fuchsin.
(3) Wash off excess of stain.
(4) Decolorise by either of the following methods :
  (*a*) Cover with, or immerse in, 1 per cent. nigrosin for five to ten minutes.
  (*b*) Cover with 5 per cent. sodium sulphite for five to thirty seconds.

(The length of time for decolorisation varies with the organism, and a large organism like the anthrax bacillus may take five times as long to decolorise as a smaller one like *B. sporogenes.*)

(5) Wash with water and dry.

(6) Place a small drop of 10 per cent. nigrosin at one end of the film and spread this in an even film over the stained specimen with the edge of another slide.

The thickness of the film depends largely on the rate of spread—the faster the spread is made the thicker is the film—and the proper thickness can be rapidly learned in a few trials.

The spores appear as clear-cut, bright red objects in the unstained bacillary body which is clearly defined by being surrounded by a perfectly homogeneous background of nigrosin.

The preliminary decolorisation, with nigrosin, sodium sulphite or other decolorising agent, need only be partial, and the final decolorisation is effected by the 10 per cent. nigrosin before it dries on the film.

STAINING OF CAPSULES

Several methods are described for general routine work. Richard Muir's method gives beautiful results, but is much more difficult for the beginner and is not recommended for general use.

HISS'S METHOD

Solutions required—

(1) Saturated alcoholic solution of gentian-violet[1]	1 part
Distilled water	19 parts
(2) Copper sulphate solution 20 per cent. in distilled water.	

Films should be thin and fixed by heat in the usual manner.

(1) Pour on the stain and gently heat until steam rises. Allow the stain to act for fifteen to twenty seconds.

(2) Wash off the stain with the 20 per cent. copper sulphate solution without washing in water, dry the film between blotting-paper, and mount, if necessary, in balsam.

In order to avoid an excessive deposit of copper sulphate crystals on the film, successive amounts of copper sulphate solution should be poured on the film until the slide is quite cool.

The bacteria are stained deep violet, while the capsules are pale violet in colour.

FLEMING'S NIGROSIN METHOD

Films are made and fixed, and a thin layer of nigrosin is spread over with a slide, as described on page 197. The slide is dried and examined. The capsule appears as a large clear space.

If the organism is Gram-positive the capsules can be demonstrated better by making a film in the usual way and staining it by Gram's method, but without using any counter-stain. The stained film is dried and a small drop of nigrosin is placed at one end of the slide, and is drawn out by means of another slide over the upper half of the film and allowed to dry.

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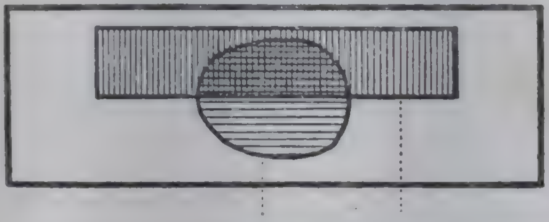
[1] Basic fuchsin may be substituted for gentian-violet.

The slide is examined with the immersion objective at the junction of the uncovered and nigrosin-covered part of the film. In one-half of the field the organism is stained purple with the Gram's stain but shows no capsule, while in the other the nigrosin has settled round the capsule which appears as a clear white space with the stained organism in the centre.



RELIEF STAINING FOR CAPSULES

(Howie and Kirkpatrick's Method)[1]

Staining solution—

10 per cent. water-soluble eosin, " yellowish or bluish," or erythrosin in distilled water .	4 parts
Serum (human, rabbit, sheep or ox heated at 56° C. for thirty minutes)	1 part
Crystal of thymol.	

Allow the mixture to stand at room temperature for several days. Centrifuge and store the supernatant fluid at room temperature ; it will keep for several months.

On a slide with a 1 mm. diameter platinum loop, mix one drop of exudate (or fluid culture, or a suspension in *broth* from an agar slope culture) with one drop of Ziehl-Neelsen's carbol-fuchsin stain diluted 1 : 5, and allow to stain for half-a-minute. Then add one drop of the eosin solution and leave for about one minute. Spread a film with cigarette paper (like a blood film). Allow to dry (do not heat), and examine in immersion oil.

In the case of Gram-positive cocci and bacilli, the preliminary staining with dilute carbol fuchsin may be omitted. Films of capsulated organisms prepared by this method show a practically homogeneous red background with an unstained capsular area prominently shown, and the bodies of the organisms stained red of about the same intensity as the background or slightly darker. The capsules are thus seen by "relief staining."

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[1] See *J. Path. Bact.*, 1934, 39, 165 ; Muir and Ritchie's *Manual of Bacteriology*, 10th edition, 124.

R. MUIR'S METHOD

Solutions required—

Strong carbol fuchsin.
Special mordant.
Löffler's methylene blue.

Mordant :

Saturated solution of mercuric chloride .	2 parts
20 per cent. solution of tannic acid . .	2 ,,
Saturated solution of potassium alum .	5 ,,

The film, which must be very thin, is dried and fixed by heat.

(1) Stain with strong carbol fuchsin for one minute, the preparation being gently heated.

(2) Wash slightly with spirit and then well in water.

(3) Pour on the mordant and allow it to act for thirty seconds.

(4) Wash well with water.

(5) Treat with spirit. The time varies with the preparation and is found by trial. About thirty to forty seconds is ample, and the film should be pale red in appearance.

(6) Wash well in water.

(7) Counter-stain with Löffler's methylene blue for a half to one minute.

(8) The preparation may be washed and dried in the usual manner,

or dehydrated with absolute alcohol, cleared in xylol and mounted in balsam. This gives somewhat clearer specimens.

The bacteria are bright red, and the capsules are bright blue.

STAINING OF FLAGELLA

Thin films are made from agar cultures. A small amount of the culture is emulsified in water, the quantity of culture being only as much as will cause the faintest turbidity of the water. A film is made from a drop of the emulsion on a clean slide and allowed to dry in the air. Strict attention should be paid to the cleanliness of the slides, which are treated with the bichromate-nitric acid solution described on p. 191, well washed, and carefully dried. They are then passed through the Bunsen flame and allowed to cool before spreading the films.

In Fleming's method, the organisms are grown on cellophane which avoids the transference of any culture medium to the slide (*vide infra*).

J. KIRKPATRICK'S METHOD[1]

Solutions required—

(1) *Fixing solution :*

Absolute alcohol	60 c.c.
Chloroform	30 c.c.
Formalin	10 c.c.

(2) *Mordant :*

Ferric chloride, 5 per cent. solution	1 part
Tannic acid, 20 per cent. solution (dissolved by heat and allowed to cool)	3 parts

Before use, dilute the mordant with an equal quantity of water.

(3) *Silver Solutions :*

(a) *Silver stock solution.*—Add 200 c.c. distilled water to 10 grams of silver sulphate[2] in a screw-capped medical flat bottle, and incubate for twenty-four hours at 37° C., shaking occasionally. This solution keeps well.

(b) *Silver staining solution.*—Rinse a clean 100 c.c. conical flask with distilled water. Place 40 c.c. of the filtered silver stock solution in the flask, and add quickly 0·6 c.c. ethylamine "33 per cent. W.V."[2] [3]. A precipitate forms which is immediately dissolved. From a clean drawn-out pipette add filtered silver stock solution until the solution remains permanently opalescent. Now add 10 c.c. distilled water.

Method

Use agar cultures thirty-six to forty-eight hours old. Heat a clean $3 \times \frac{1}{2}$ in. test-tube and allow to cool. Suspend a loopful of the agar culture in 1 c.c. sterile distilled water in the tube, rotating the loop very gently. Add distilled water until the suspension is faintly opalescent.

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[1] Method described in 10th edition of Muir and Ritchie's *Manual of Bacteriology.*

[2] British Drug Houses Ltd.

[3] W.V. = Weight-Volume—*i.e.* 33 per cent. by weight of ethylamine.

(*a*) Place a loopful of the suspension on a clean slide and draw the excess to one side by means of the platinum wire, so that a thicker part of the film (which acts as a control spot in the staining process) is formed. Allow the film to dry in the air.

(*b*) Treat the film with the fixing solution for one to three minutes.

(*c*) Rinse in spirit and wash thoroughly in water.

(*d*) Treat with the mordant for three to five minutes.

(*e*) Wash well with water and dry the under surface of the slide.

(*f*) Filter on the silver staining solution and heat gently until the thick control spot becomes dark brown in colour, and a metallic scum appears on the edges of the fluid—about fifteen seconds. Cease heating and allow the solution to act for a further fifteen to thirty seconds.

(*g*) Wash off the staining solution in running water (do not pour off).

(*h*) Dry the film, and mount in balsam.

If the staining is successful the organisms are stained black, while the flagella are clearly defined and are light brown or grey in colour. A granular appearance in the flagella may be due to excess of ethylamine, over-heating, or over-treatment in the mordant. Too little ethylamine or excessive heating may cause a crystalline deposit. If the directions are carefully followed the method gives good results.

FLEMING'S METHOD FOR THE DEMONSTRATION OF FLAGELLA

A cellophane disk sterilised in distilled water in the autoclave is placed on the surface of an agar plate. This is dried off in the incubator for a short time. The surface of the cellophane is then inoculated with the organism, *B. typhosus*, *B. coli*, *B. proteus*, etc., which is grown for a period not exceeding twenty-four hours. By means of forceps the cellophane disk is removed, together with the whole of the culture, and transferred to another Petri dish after which about 20 c.c. of sterile distilled water is added (sufficient to cover the disk). This is incubated for a few hours to allow the bacteria to float off into the water. Then 1 c.c. of formalin is very gently added to fix the bacteria, and the plate is left overnight undisturbed to allow fixation to take place, with the minimum disturbance and separation of flagella. The suspension is poured into a screw-capped bottle and preserved. This suspension keeps perfectly well for a long time.

For use remove sufficient from the upper part of the tube and add to distilled water.

Technique—

Slides.—It is usually stated that slides must be specially cleaned, but ordinary cleansing is quite satisfactory with this method.

Making films.—A drop of a thin suspension is placed on the slide and spread over a large part of the slide with a wire or pipette. This is best allowed to dry at room temperature.

Mordant.—Stock solutions of 20 per cent. tannic acid and 5 per cent. tartar emetic can be kept. (20 per cent. tannic acid is convenient as this can also be used for Kirkpatrick's method.)

To three parts of 20 per cent. tannic acid add two parts of 5 per cent. tartar emetic and five parts of water. (A heavy precipitate forms which dissolves on boiling.) This is heated to boiling point in a test-tube and the slide is flooded with it. After two minutes wash well with tap water.

Silver solution.—A saturated solution of silver sulphate is diluted with an equal volume of distilled water and ethylamine is added until the precipitate is just re-dissolved. This solution keeps indefinitely.

This is heated not quite to boiling point and the slide is flooded. Allow to act without further heating for about one minute, then wash off rapidly with tap water. The slide may then be blotted and dried in the usual way.

To make permanent preparations the silvered slide is immersed for half to one hour in weak gold chloride solution.

The features of this method are :

No culture medium is present in the suspension except the minute amount on the cellophane disk.

The bacteria are fixed with the minimum disturbance so that their flagella are preserved intact, while the formalin appears to assist the staining.

The silver ethylamine solution keeps for a long time and is always ready for use.

The method is essentially an improved and simpler modification of Zettnow's method and is very suitable for class purposes.

THE ROMANOWSKY STAINS

The original Romanowsky stain was made by dissolving in methyl alcohol the compound formed by the interaction of watery solutions of eosin and zinc-free methylene blue. The original stain has now been replaced by various modifications which are easier to use and give better results; these are: Leishman's, Wright's, Jenner's and Giemsa's stains. The peculiar property of the Romanowsky stains is that they impart a reddish-purple colour to the chromatin of malaria and other parasites. This colour is due to a substance which forms when methylene blue is "ripened," either by age, as in polychrome methylene blue, or by heating with sodium carbonate. The latter method is employed in the manufacture of Leishman's and Wright's stains. The ripened methylene blue is mixed with a solution of water-soluble eosin, when a precipitate, due to the combination of these dyes, is formed. The precipitate is washed with distilled water, dried, and dissolved in pure methyl alcohol.[1] Each modification of the Romanowsky stain varies according to the "ripening" and the relative proportions of methylene blue and eosin.

According to the nature of the microscopic preparation, different stains are employed. Thus for the cytological examinations of blood, Jenner's stain is used; for the malaria parasite and trypanosomes, Leishman's and Wright's modifications give the best results, while the pathogenic spirochaetes (particularly the *Treponema pallidum* of syphilis) and certain protozoa can be demonstrated best by Giemsa's stain.

The Romanowsky stains, except Jenner's stain, are usually diluted for staining purposes with distilled water, when a precipitate is formed which is removed by subsequent washing.

JENNER'S STAIN

This can be purchased ready for use, but may be made by dissolving 0·5 gram of powdered stain in 100 c.c. pure methyl alcohol. This stain is satisfactory for the cytological examination of blood films, but is not so suitable for parasites.

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[1] The methyl alcohol must be "pure, for analysis," and have a pH of 6·5. If too acid, the reaction must be adjusted (with the usual indicators) by the addition of N/100 NaOH.

The stain is poured on the dried but *unfixed* film and allowed to act for one to three minutes. The film is then washed with distilled water until pink. The slide is blotted and allowed to dry in the air.

LEISHMAN'S STAIN

This stain may be purchased ready for use or made by dissolving 0·15 gram of Leishman's powder in 100 c.c. pure methyl alcohol. The powder is ground in a mortar with a little methyl alcohol, the residue of undissolved stain allowed to settle and the fluid decanted into a bottle. The residue in the mortar is treated with more methyl alcohol, and the process is repeated until all the stain goes into solution. The remainder of the methyl alcohol is now added. The stain is improved by keeping two weeks before use.

Films.—Dry unfixed films are used. The stain is first used undiluted, and the methyl alcohol fixes the film. The stain is then diluted with distilled water, and the staining proper carried out.

(1) Pour the undiluted stain on the unfixed film and allow it to act for two minutes.

(2) By means of a pipette and rubber teat add double the volume of distilled water to the slide, mixing the fluids by alternately sucking them up in the pipette and expelling them. Allow the diluted stain to act for five to seven minutes.

(3) Wash the slide gently with distilled water, allowing the preparation to differentiate in the distilled water until the film appears bright pink in colour—usually about a minute.

(4) Remove the excess of water with blotting-paper and dry in the air.

It is important that the reaction of the distilled water be neither acid nor alkaline. Any slight variations from neutrality may alter considerably the colour of granules in white blood corpuscles, etc., and give rise to supposed "pathological" appearances in cells which are really normal. A simple method of ensuring a suitable reaction of the distilled water is to keep large bottles of it—*e.g.* aspirator bottle—specially for these stains. Add 2 or 3 drops of 1 per cent. aqueous neutral-red solution. The usual reaction of distilled water is slightly acid, and a few drops of 1 per cent. sodium carbonate solution should be added until the solution shows the faintest possible suggestion of pink colour.

When the staining is excessively bluish, as in old films, good differentiation is obtained by brief washing with 1 per cent. mono-sodium phosphate.

Sections—

(1) The section is treated with xylol to remove the paraffin, then with alcohol, and finally distilled water.

(2) Drain off the excess of water and stain for five to ten minutes with a mixture of 1 part stain and 2 parts of distilled water.

(3) Wash with distilled water.

(4) Differentiate with a weak solution of acetic acid (1:1500), controlling the differentiation under the low power of the microscope until the protoplasm of the cells is pink and only the nuclei are blue.

(5) Wash with distilled water.

(6) Blot, dehydrate with a few drops of absolute alcohol, clear in xylol and mount in Canada balsam or preferably D.P.X. mounting medium (p. 194).

> *Note.*—If the eosin tint is too pronounced it can be lightened by the use of very dilute caustic soda solution (1 : 7000) which is washed off whenever the desired colour has been obtained.

J. H. WRIGHT'S STAIN

This is similar to Leishman's stain and is used more in America than in this country. It should be purchased ready for use. The method of staining is, for all practical purposes, the same as for Leishman's stain.

GIEMSA'S STAIN

This consists of a number of compounds made by mixing different proportions of methylene blue and eosin. These have been designated Azur I, Azur II and Azur II-eosin. The preparation is best purchased made up, *e.g.* by G. T. Gurr, London, or British Drug Houses Ltd., but may be prepared as follows :—

Azur II-eosin	3 grams
Azur II	0·8 gram
Glycerol (chemically pure)	250 grams
Methyl alcohol (acetone-free)	250 grams

The stain may be used in a manner similar to Leishman's preparation (the "rapid method"), or prolonged staining may be carried out, as, for example, in staining spirochaetes (the "slow method"). In both cases the preparation must be fixed prior to staining, either by methyl alcohol for three minutes, or by absolute alcohol for fifteen minutes.

RAPID METHOD

(1) Fix films in methyl alcohol—three minutes.

(2) Pour on a mixture of 1 part stain and 2 parts distilled water (*vide* p. 217) and allow to act for five minutes.

(3) Wash with distilled water, allowing the preparation to differentiate for about half-a-minute.

(4) Blot and allow to dry in the air.

A rapid method with the application of heat is useful for demonstrating spirochaetes :—

Fix preparations with absolute alcohol (fifteen minutes) or by drawing three times through a flame. Prepare a fresh solution of 10 drops of Giemsa's solution with 10 c.c. of distilled water of correct pH (p. 218), shake gently, and cover the fixed film with the diluted stain. Warm till steam rises, allow to cool for fifteen seconds, then pour off and replace by fresh stain and heat again. Repeat the procedure four or five times, wash in distilled water, dry and mount.

SLOW METHOD

This is a specially valuable method for demonstrating objects difficult to stain in the ordinary way, *e.g.* certain pathogenic spirochaetes. The principle is to allow the diluted stain to act for a considerable period. As the mixture of stain and water causes a fine precipitate, care has to be taken that this does not deposit on the film.

Cover-slips.—The film is fixed in methyl alcohol for three minutes. A mixture is made in a Petri dish in the proportion of 1 drop of stain to 1 c.c. of distilled water. The cover-slip, when fixed and still wet with the alcohol, is placed carefully, film downwards, on the surface of the mixture. When properly done, the cover-slip remains floating. The lid is carefully placed on the Petri dish and the stain allowed to act overnight. The cover-slip is then washed in a stream of distilled water, allowed to dry in the air, and mounted. There should be no deposit of precipitated stain on the preparation.

Slides.—The film is fixed in methyl alcohol for three minutes as with cover-slips. The mixture of stain and distilled water is made in a large (6 in.) Petri dish if there are several slides to stain. A piece of thin glass rod is placed in the Petri dish, and the slides, after fixing, are laid film downwards in the fluid with one end of the slide resting on the glass rod so that there is sufficient staining fluid between the film and the bottom of the dish.

A sheet of glass slightly curved is also convenient; the slide with the film downward lies across the concavity, the space between containing diluted stain. The curved plate and slides should be placed in a box with a close-fitting lid to avoid evaporation.

After sixteen to twenty-four hours' staining, the slides are washed and dried as in the case of cover-slips.

Adachi's Modification.—This method has been utilised for staining the flagella of *Spirillum minus* (*vide* p. 556) and can also be applied in the staining of delicate spirochaetes. The preparation is fixed for thirty to sixty seconds, by osmic acid vapour over the following solution: osmic acid, 1 gram, distilled water, 100 c.c., 10 drops of 5 per cent. mercuric chloride; and then stained overnight in dilute Giemsa's solution (*vide supra*) to each 10 c.c. of which 0·6 c.c. of 1 per cent. potassium carbonate has been added.

FONTANA'S METHOD OF STAINING SPIROCHAETES

Solutions required—

(a) *Fixative :*

Acetic acid	1 c.c.
Formalin	2 c.c.
Distilled water	100 c.c.

(b) *Mordant :*

Phenol	1 gram
Tannic acid	5 grams
Distilled water	100 c.c.

(c) *Ammoniacal silver nitrate :*

Add 10 per cent. ammonia to 0·25 per cent. solution of silver nitrate in distilled water until the precipitate formed just dissolves. Now add more silver nitrate solution drop by drop until the precipitate returns and does not re-dissolve.

Method—

(1) Treat the film three times, thirty seconds each time, with the fixative.

(2) Wash off the fixative with absolute alcohol and allow the alcohol to act for three minutes.

(3) Drain off the excess of alcohol and carefully burn off the remainder until the film is dry.

(4) Pour on the mordant, heating till steam rises, and allow it to act for half-a-minute.

(5) Wash well in distilled water and again dry the slide.

(6) Treat with ammoniacal silver nitrate, heating till steam rises, for half-a-minute, when the film becomes brown in colour.

(7) Wash well in distilled water, dry and mount in Canada balsam.

It is essential that the specimen be mounted in balsam under a cover-slip before examination, as some immersion oils cause the film to fade at once.

The spirochaetes are stained brownish-black on a brownish-yellow background.

BECKER'S METHOD OF STAINING SPIROCHAETES

Dried, thin, unfixed films should be used. Treat for one minute with the same fixative as used in Fontana's method, the fluid being renewed twice during this time. After washing in water (half-a-minute) treat with the same mordant as used in Fontana's method for three to five minutes.

After rinsing well in water, place in the following staining solution for three to five minutes :

Basic fuchsin, saturated alcoholic solution		45 c.c.
Shunk's mordant—		
95 per cent. alcohol	16 c.c.	18 c.c.
Aniline	4 c.c.	
Distilled water		100 c.c.

When making up this stain, thoroughly dry the glass-ware or rinse it well with alcohol. Add the Shunk's mordant to the alcoholic fuchsin solution, mix, and add the distilled water. Filter before use.

Wash well in water, dry, and mount in balsam.

LEVADITI'S METHOD OF STAINING SPIROCHAETES IN TISSUES

(*a*) ORIGINAL METHOD

1. Fix the tissue, which must be in small thin pieces (1 mm. thick), in 10 per cent. formalin for twenty-four hours.

2. Wash the tissue for one hour in water and thereafter place it in 96–98 per cent. alcohol for twenty-four hours.

3. Transfer to 1·5 per cent. silver nitrate solution in a dark bottle and keep in the incubator for three days.

4. After washing in water for twenty to thirty minutes, place the piece of tissue in the reducing mixture, made up as follows :—

Pyrogallic acid	4 grams
Formalin	5 c.c.
Water	100 c.c.

and allow it to remain in this mixture in a dark bottle for forty-eight hours at room temperature.

5. After washing well with water, dehydrate the tissue with increasing strengths of alcohol and embed in paraffin (see p. 230). Thin sections are cut

and mounted in the usual way. After removing the paraffin with xylol the sections are immediately mounted in Canada balsam.

(*b*) PYRIDINE METHOD

This method is more rapid, but in our experience does not give quite such good results as the older method :

1. Fix the tissue in formalin as in the other method, harden in alcohol overnight and then wash in distilled water.
2. Place the tissue in a 1 per cent. solution of silver nitrate (to which one-tenth of the volume of pure pyridine has been added) for two hours at room temperature, and thereafter at about 50° C. for four to six hours. It is then rapidly washed in 10 per cent. pyridine solution.
3. Transfer to the reducing fluid, which consists of :

Formalin 4 per cent..	100 parts
to which are added immediately before use :	
Acetone (pure)	10 ,,
Pyridine (pure)	15 ,,

Keep the tissue in this fluid for two days at room temperature in the dark.

4. After thorough washing, the tissue is dehydrated, embedded, cut and mounted in the usual way.

JAHNEL'S METHOD OF STAINING SPIROCHAETES IN TISSUE

1. Wash in water, for one to three days, thin pieces 2–4 mm. thick, which have been *well fixed* in formalin.
2. Place in pure pyridine for three days.
3. Wash in many changes of water during a period of two to three days until the pyridine almost disappears. Then allow the pieces to remain a few days in 5–10 per cent. formalin.
4. Place in water again for two days.
5. Treat with uranium nitrate (Merck) 1 per cent. solution in distilled water, for half to one hour in the incubator at 37° C.. The tissue should rest on lead-free glass-wool to aid penetration.
6. Wash out in distilled water for one day.
7. Allow to remain in 96 per cent. alcohol, three to eight days.
8. Wash out in distilled water until the block sinks.
9. Place the tissue in a dark flask and treat it with a freshly

prepared silver nitrate solution 1·5 per cent., for five to eight days in the incubator at 37° C..

10. Decant the silver nitrate solution, wash the tissue in water, and transfer to the following developer for one to two days :

Pyrogallol 4 per cent.	90 c.c.	of this reject 15 c.c. and replace by pyridine.
Acetone	10 c.c.	

11. Wash in distilled water ; embed in paraffin in the usual manner.

STAINING OF AMOEBAE AND OTHER INTESTINAL PROTOZOA IN FAECES

Wet smears should be fixed in a mixture of

Alcohol	1 part
Saturated aqueous solution of mercuric chloride	2 parts

for five minutes or longer.

The films are then washed in 50 per cent. spirit and treated with Gram's iodine for two minutes, to remove the mercury salt, the iodine being removed with spirit and the films washed.

Stain with iron haematoxylin for ten to twenty minutes.

Iron Haematoxylin :

(*a*)	Haematoxylin	1 gram
	Absolute alcohol	100 c.c.
(*b*)	Liquor ferri perchlor. 30 per cent.	4 c.c.
	Concentrated hydrochloric acid	1 c.c.
	Distilled water	100 c.c.

Mix equal parts of (*a*) and (*b*) immediately before using.

After staining, wash films in water, pass through alcohol, clear with xylol and mount in balsam, as in the treatment of tissue sections.

Preparations may be counter-stained with Van Gieson's stain, fifteen to thirty seconds :

Saturated aqueous solution of acid fuchsin	1–3 parts
Saturated aqueous solution of picric acid	100 ,,

Dehydrate rapidly with absolute alcohol, clear in xylol and mount in balsam.

Note.—Fixed wet preparations must be treated in the same manner as sections and never allowed to become dry.

STAINS FOR INCLUSION BODIES AND ELEMENTARY BODIES IN VIRUS INFECTIONS, AND FOR RICKETTSIAE

INCLUSION BODIES

For intranuclear and cytoplasmic inclusions Giemsa's stain, p. 218, is satisfactory when such forms are of a basophilic nature as in psittacosis. For acidophile inclusion bodies other stains give more satisfactory results.

Mann's Methyl-Blue Eosin Stain.

1 per cent. aqueous solution of methyl-blue	35 parts
1 ,, ,, ,, ,, ,, eosin	45 ,,
Distilled water	100 ,,

Tissues should be fixed in Bouin's solution (p. 230) or Zenker's fluid (p. 228), and paraffin sections cut in the usual way. Stain for 12 hours in the incubator at 37° C.. The section is then rinsed in water, differentiated under the microscope in 70 per cent. alcohol to each c.c. of which has been added one drop of saturated aqueous Orange G. solution, dehydrated and mounted in balsam.

In Ford's modification the sections are stained for 3 hours at 37° C., treated with 40 per cent. formaldehyde (strong formalin) for five seconds, washed in water, differentiated and mounted as above. This method is especially useful for staining the Negri bodies in rabies.

ELEMENTARY BODIES

Giemsa's Stain.

This has already been described on p. 218, and while satisfactory for the elementary bodies of vaccinia and psittacosis, it has been replaced by other methods which are quicker, free from deposit, and give more consistent results.

Paschen's Method.

This method is especially recommended. Films are prepared from the infected tissues, etc., on glass slides of a thickness suitable for critical high-power microscopical investigation (p. 62), and allowed to dry. They are placed in distilled water for five minutes, dried in air, covered or immersed in absolute alcohol for five minutes and again dried in air. They are treated with Löffler's flagella mordant:

Tannic acid, 20 per cent. aqueous solution	100 c.c.
Ferrous sulphate, saturated aqueous solution	50 c.c.
Basic fuchsin, saturated alcoholic solution	10 c.c.

This is gently heated for one minute and allowed to remain on the slide for ten minutes. The mordant is washed off with distilled water and the film stained with carbol fuchsin solution, 1 part in 20 of distilled water, gentle heat being applied for a minute. The film is then rapidly washed with water, dried between blotting-paper, and mounted in immersion oil.

Castaneda's Method.

This method is also particularly useful for Rickettsiae. A buffer formaldehyde solution is prepared as follows :—

1 gram of monopotassium dihydrogen phosphate is dissolved in 100 c.c. of distilled water ; 25 grams of disodium hydrogen phosphate (Na_2HPO_4, 12 H_2O) are dissolved in 900 c.c. distilled water ; the two solutions are mixed so that the pH is 7·5. 1 c.c. of strong formalin is added as a preservative.

The stain consists of a 1 per cent. solution of methylene blue in methyl alcohol.

20 c.c. of buffer solution are mixed with 1 c.c. formalin, and 0·15 c.c. of the methylene blue solution. The mixture is applied to the film for three minutes and then decanted without washing. Counter-stain for one or two seconds with

Safranin "O," 0·2 per cent. aqueous solution .	1 part
Acetic acid, 0·1 per cent. ,, ,, .	3 parts

Wash in running water, blot and dry.

The rickettsiae remain blue while the protoplasm and nuclei of the cells are red.

This stain can also be used for elementary bodies, *e.g.* those of psittacosis. It may be modified by using Azur II in place of methylene blue.

IMPRESSION PREPARATIONS

These have been used in the morphological study of the pleuropneumonia group of organisms[1] and of "rough" and "smooth" colonies of various bacteria.[2]

The essential part of the technique is to remove a small slab about 2 mm. thick of the solid medium (*e.g.* serum-agar) on which the organism is growing and place it colony downwards on a cover-slip. The whole is immersed in fixative, so that the fixing fluid penetrates through the agar to reach the colony. When the bacteria are fixed, the agar is removed carefully from the cover-slip which is well washed for two hours in distilled water, suitably stained and mounted. As fixative

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[1] See Klieneberger, E., *J. Path. Bact.*, 1934, **39**, 409.

[2] See Bisset, K. A., *J. Path. Bact.*, 1938, **47**, 223.

Bouin's fluid (p. 230) may be used, or Flemming's solution.[1] For staining, methylene blue or dilute carbol fuchsin may be employed, but Giemsa's stain, applied by the slow method (p. 219), is the most satisfactory for the pleuropneumonia organism. The agar slabs, after fixation, may also be embedded and vertical sections of the colony cut with a microtome.

FIXATION AND EMBEDDING OF TISSUES; SECTION CUTTING

As the ordinary routine bacteriological investigation of tissues is carried out almost exclusively with paraffin sections, this technique only will be described.

The fixed tissue is embedded in paraffin wax to support it during the cutting of the section, and the section is held together by the wax in the process of transferring it to the slide.

The paraffin wax must completely permeate the tissue, but before it can do so, all water must be removed from the material and replaced by a fluid with which melted paraffin will mix.

Water, therefore, is first removed by several changes of alcohol; the alcohol is replaced by some fluid—such as benzol, xylol, acetone, chloroform—which is a solvent of both alcohol and paraffin wax, and the tissue is finally embedded in melted paraffin.

Before removing the water from the tissue preparatory to embedding, the tissue must be suitably fixed and hardened.

The essentials for obtaining good sections are:

(1) The tissue must be fresh.

(2) It must be properly fixed by using small pieces and employing a large amount of fixing fluid.

(3) The appropriate fixing fluid must be employed for the particular investigation required.

(4) The tissue must not remain too long in the embedding bath.

FIXATIVES

FORMALIN

Ten per cent. commercial formalin in normal saline solution is a good fixative for general use. Its advantages are: it is easily prepared, has good penetrating qualities, does not shrink the tissues, and permits considerable latitude in the time during which specimens may be left in it. Moreover the subsequent handling of the material is much easier in our experience than in the case of mercuric chloride fixatives, such as Zenker's fluid. Formalin fixation is not so good as other methods where fine detail has to be observed, as, for example, in material containing protozoa. For general routine use, however, it is the most convenient and useful of fixatives. Tissue should be cut into thin slices, about $\frac{1}{8}$-in. thick, and dropped into a large bulk of fixative. The fluid may be changed at the end of twenty-four hours, and fixation is usually complete in forty-eight hours. Specimens are then washed in running water for an hour and transferred to 50 per cent. spirit. In the latter fluid they may be kept for a considerable time without deterioration.

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[1] Osmic acid, 0·1 per cent.; chromic acid, 0·2 per cent.; glacial acetic acid, 0·1 per cent. The osmic and chromic acids, when mixed, will keep only for three to four weeks, while the acetic acid should only be added immediately before use.

Formalin tends to become acid owing to the formation of formic acid. The strong formalin should be kept neutral by the addition of excess of magnesium carbonate. The clear supernatant fluid is decanted off when formalin dilutions are required.

ZENKER'S FLUID

Mercuric chloride	5 grams
Potassium bichromate	2·5 grams
Sodium sulphate	1 gram
Water	100 c.c.

Immediately before use, 5 c.c. of glacial acetic acid per 100 c.c. of fluid are added.

The fluid should be warmed to body temperature and only small pieces of tissue must be placed in it. Fixation is complete in twenty-four hours, and thereafter the pieces of tissue are washed in running water for twenty-four hours to remove the potassium bichromate and mercuric chloride. The tissue is then transferred to 50 per cent. spirit.

It is essential that all the mercuric chloride should be removed, otherwise a deposit will appear in the sections. The bulk of it is removed by washing. The remainder can be removed with iodine during the dehydration stage in alcohol. The material after washing is transferred to 50 per cent., and later to 70 per cent. alcohol to which sufficient iodine has been added to make the fluid dark brown in colour. (It is convenient to keep a saturated solution of iodine in 90 per cent. alcohol in a drop bottle, and add a few drops as required.) If the alcohol becomes clear more iodine is added until the fluid remains brown. This indicates that all the mercury salt has been dissolved out by the iodine-alcohol.

Cut sections fixed on slides can also be treated with iodine—*e.g.* Gram's iodine—for three to five minutes, to remove mercuric chloride.

Animal tissues fixed in Zenker's fluid are more difficult to cut, and sections are apt to float off the slide, particularly if fixation has been unduly prolonged.

ZENKER-FORMOL FLUID

This is similar to Zenker's fluid except that the acetic acid is omitted and 5 c.c. of formalin are added per 100 c.c. immediately before use. It is a useful general fixative for animal tissues.

MERCURIC CHLORIDE-FORMALIN SOLUTION

Mercuric chloride, saturated aqueous solution	90 c.c.
Formalin, commercial	10 c.c.

Small portions of tissue must be used and fixation is complete in one to twelve hours. Then transfer to alcohol and iodine as after Zenker's fluid (*q.v.*). This fluid fixes with the minimum amount of distortion and the finer cytological details of the cells are retained.

"SUSA" FIXATIVE (M. HEIDENHAIN)

Mercuric chloride	45 grams
Distilled water	800 c.c.
Sodium chloride	5 grams
Trichloracetic acid	20 grams
Acetic acid (glacial)	40 c.c.
Formalin (40 per cent. formaldehyde) .	200 c.c.

This is one of the best fixatives for both normal and pathological histology. Tissues not thicker than 1 cm. should be fixed for three to twenty-four hours, depending on the thickness. The material should be transferred *direct* to 96 per cent. alcohol. Lower grades of alcohol, or water, may cause undue swelling of connective tissue. Add to the alcohol sufficient of a saturated solution of iodine in 96 per cent. alcohol to give a brown colour. If the latter fades, more iodine should be added.

The advantages of "Susa" fixative are rapid and even fixation with little shrinkage of connective tissue. The transference direct to 96 per cent. alcohol shortens the time of dehydration, while tissues thus fixed are easy to cut.

BOUIN'S FLUID

This fixative is useful for the investigation of inclusion bodies.

Saturated aqueous solution of picric acid .	75 parts
Formalin	25 ,,
Glacial acetic acid	5 ,,

This solution keeps well. Use thin pieces of tissue not exceeding 10 mm. thick. Fix for 1–12 hours according to thickness and density of tissue. Wash in 50 per cent. alcohol (not water), then 70 per cent., until the picric acid is removed.

EMBEDDING AND SECTION CUTTING

After fixation by any of the previous methods and transference to 50 per cent. alcohol, *small pieces* of tissue are treated as follows :—

(1) Place in 90 per cent. spirit for two to five hours.

(2) Transfer to absolute alcohol for two hours.

(3) Complete dehydration in fresh absolute alcohol for two hours.

(4) Transfer to a mixture of absolute alcohol and chloroform (equal parts) till tissue sinks, or overnight.

(5) Place in pure chloroform for six hours.

(6) Transfer the tissue for one hour to a mixture of equal parts of chloroform and paraffin wax, which is kept melted in the paraffin oven.

(7) Place in pure melted paraffin in the oven at 55° C. for two hours, preferably in a vacuum embedding oven.

The tissue is embedded in blocks of paraffin. These are cut out, trimmed with a knife, and sections 5μ thick are cut by means of a microtome. The sections are flattened on warm water, floated on to slides and allowed to dry. Albuminised slides are useful where the staining process involves heating, and where animal tissue is used, especially after fixation with Zenker's fluid. The slides are coated with albumin either by means of a small piece of chamois leather or by the finger. The albumin solution is made by adding three parts of distilled water to one part of egg-white and shaking thoroughly. The mixture is filtered through muslin into a bottle, and a crystal of thymol is added as a preservative. It is usual to coat a number of slides, and, after drying, the slides are stored until required. The albuminised side may be identified by breathing gently on the slide; it is not dimmed by the breath, whereas the plain side is.

For further treatment of sections, see Staining Methods.

For additional details, reference must be made to works on histology.

CHAPTER VII

ANIMAL INOCULATION AND AUTOPSY

In Great Britain animal experiments may be performed only under a licence granted by the Home Secretary. In addition to the licence various certificates have also to be obtained, depending on the nature of the experiments and the animals used.

The usual animals employed for bacteriological experiments are the guinea-pig, rabbit, mouse and white rat, and *the commonest method of inoculation is by means of a hypodermic needle and syringe.* According to the method of inoculation and the size of the animal, the amount of injected material varies, and a number of syringes of different sizes are used. A convenient "battery" of syringes is the following: 1 c.c. "tuberculin" syringe graduated to 0·01 c.c.; 2 c.c. syringe graduated to 0·1 c.c.; 5 c.c. graduated to 0·25 c.c.; 10 c.c. graduated to 0·5 c.c. A 20 c.c. syringe is used only occasionally. Syringes may be of the "Record" type, or of the all-glass "Luer" type. A selection of needles is required, of which the following are useful: fine-bore, No. 25 gauge; medium-bore for general use, Nos. 21 and 22 gauge; large-bore, Nos. 16–18 gauge, for inoculating thick suspensions or emulsions of tissue. The needles should be made from stainless steel.

Syringes are sterilised by taking them apart, placing in cold water, and boiling for ten to fifteen minutes. All-glass syringes may conveniently be sterilised ready for use as follows. The needle is fitted and the plunger lightly smeared with liquid paraffin and inserted into the barrel. The assembled syringe is placed in a test-tube longer than itself, and of such a bore that the shoulder of the barrel rests on the rim. The shoulder is wrapped with a little gauze or kraft paper so that it does not rest directly on the glass. The whole is wrapped in kraft paper and sterilised in the hot-air oven.[1] The syringe remains sterile indefinitely in its wrapping and is always ready for immediate use.

After use it should be washed out with weak lysol. If a " Record " syringe, it is again boiled, then dried and put away with the piston and barrel separate, or a small quantity of liquid paraffin is smeared over the plunger before it is fitted into the barrel. If an all-glass syringe, it is washed with water, wiped with methylated spirit, and finally coated over with liquid paraffin. It is then put up in a test-tube, as above described, and sterilised. Needles may be stored in alcohol or dried and a little alcohol run through them. The stilette should be lightly smeared with vaseline before replacing it in the needle. Needles should always be kept sharp and the points renewed on a fine oil-stone (Arkansas slip-stone).

MATERIAL INOCULATED

Urine, *cerebro-spinal fluid*, *blood*, and *serous fluids* are easily inoculated with a medium-bore needle. Tenacious material such as *pus* and *sputum* is injected through a wide-bore needle.

Cultures.—Fluid cultures are easily drawn through a medium-bore needle. It will be found advantageous first to pour the culture into a small (2 in.) Petri dish, or a 50 c.c. conical test-glass. Growths on solid media may be scraped off and suspended in broth or saline, or the diluting fluid may be poured on the culture, which is then emulsified with a wire loop.

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[1] " Record " syringes must never be sterilised in the hot-air oven, as the heat will melt the cement or solder which unites the glass barrel with the metal parts.

Tissues.—These should be cut into small pieces in a sterile porcelain mortar by means of scissors sterilised by boiling. Some clean coarse sand, contained in a stoppered bottle, and sterilised by hot air, is then added to the mortar and the whole thoroughly ground with the pestle. When the tissue has been well ground up, saline is added, and the mixture further triturated. On standing for a short time, the sand and tissue rapidly settle to the bottom of the mortar, and the supernatant fluid can be drawn into the syringe. When intravenous inoculation of tissue suspension has to be employed, care must be taken that no large particles are injected. To avoid this, the suspension must be centrifuged at low speed, and only the supernatant fluid used.

GUINEA-PIGS

These animals vary in size, and weigh from 200 grams (small) to 1000 grams (large). A good average weight for general purposes is 400 grams.

METHODS OF INOCULATION

Subcutaneous.—An assistant holds the animal during the operation, and the injection is made under the skin of the flank. The animal is grasped across the shoulders in one hand, with the thumb curved roun the animal's neck so that it rests on the lower jaw The hind legs are secured between the first and second and second and third, fingers of the other hand, the knuckles being uppermost, and the animal is held s that the flank is presented for inoculation. The ski may be disinfected with tincture of iodine, but it i not necessary to do so. The operator picks up a fol of skin and introduces the point of the needle int the base of the fold, so that it lies in the subcutaneous tissue. Amounts up to 5 c.c. can be introduced. A 2 c.c. or a 5 c.c. syringe is convenient for the purpose.

Some workers inoculate by picking up a fold of skin about the mid-abdomen. A needle is introduced into the base of the fold and passed down in the subcutaneous tissue until it reaches the groin where the injection is made. This method obviates superficial ulceration when tuberculous material is injected.

Intraperitoneal.—The animal is held in a similar manner. The inoculation is made in the mid-line in the lower half of the abdomen. The skin may be sterilised. The assistant holds the animal with its head downwards, so that the intestines fall towards the diaphragm. The skin is pinched up, the point of the needle passed into the subcutaneous tissue, and then downwards through the abdominal wall into the peritoneal cavity. There is no risk of damage to the intestines. Not more than 5 c.c. can safely be inoculated intraperitoneally.

Intracutaneous.—This method is used chiefly in testing cultures of the diphtheria bacillus for virulence (*vide* p. 380). The hair is removed from the flanks of the animal by shaving or by means of a fresh 5 per cent. solution of sodium sulphide or a depilating powder. White guinea-pigs (300–400 grams weight) are used, as the skin is unpigmented and the results of the test can easily be read.

The depilating powder is made as follows :—

Barium sulphide, commercial powder	7 parts
White household flour	7 parts
Talcum powder	7 parts
Castile soap powder	1 part

Remove the hair from the flanks as closely as possible with hair clippers. Make up the depilating powder into a smooth paste with water, and rub into the animal's hair with a wooden spatula or toothbrush. Allow the paste to act for one minute and renew the application. After two minutes remove the paste with the spatula or handle of the toothbrush. Now wash the animal's skin and surrounding hair with warm water and dry with a cloth. The depilated surface should be quite smooth and white, and it is not advisable to leave the paste on too long as the skin becomes red and excoriated in patches, making the subsequent observation of reactions very difficult. The depilating powder should be used at least one hour before the intracutaneous injection is carried out.

For the test a 1 c.c. all-glass tuberculin syringe, fitted with a short needle of 25 or 26 S.W.G. (exactly as used for Schick and Dick tests), is employed. The skin of the animal is pinched up between the thumb and forefinger, and the point of the needle is inserted at the top of the fold so that the bevel of the needle is towards the surface of the skin. The needle passes only into the dermis, as near the surface as possible, and not into the subcutaneous tissue. 0·2 c.c. is the amount usually used, and when several tests are to be made the injections should be about one inch apart and not too near the middle line of the abdomen. No more than ten injections should be made on one animal. The results are read twenty-four to forty-eight hours later.

The normal rectal temperature of the guinea-pig is $100{\cdot}8 \pm 1{\cdot}2^{\circ}$ F.. To ascertain the animal's temperature, a clinical thermometer, with a small round bulb which is smeared with vaseline, is gently inserted into the rectum of the animal.

RABBITS

These animals are often unsatisfactory for experimental purposes owing to their liability to parasitic and intercurrent infections. The animals used should be free from snuffles (a chronic nasal inflammation), subcutaneous abscesses, and mange. They should be plump, their fur should be in good condition, and they should not be suffering from diarrhoea. If the animal is in poor condition it is probably affected with coccidiosis or intestinal worms. Rabbits are very prone to die from septicaemia (due to *B. lepisepticus*), and from pneumonia. It is better to use animals obtained from a reliable breeder, where the condition of the stock is known, than to purchase rabbits casually and indiscriminately from a dealer.

The normal rectal temperature of the rabbit is 102·4° F., but the variations are great. No temperature under 104° F. should be considered pathological. The leucocyte count of the rabbit is also subject to great normal variation.

The chief use of the rabbit lies not so much in diagnostic work as in its value for experimental purposes. It is extensively used for the production of immune sera, such as agglutinating and haemolytic sera, which are frequently employed for routine laboratory diagnosis.

Under Certificate "A" of the Home Office the animal may be inoculated intravenously, intraperitoneally, subcutaneously, or by scarification, without the use of an anaesthetic.

Scarification.—The hair is removed from the flank of the animal by first clipping and then shaving, or by means of the depilating mixture described on p. 235. The skin is cleansed with alcohol, which is allowed to evaporate. A number of parallel scratches are made with a sharp sterile scalpel, just sufficiently deep to draw blood. The infective material is rubbed into the scarified area by means of a platinum loop or the side of the scalpel. This method is mainly used for the propagation of vaccinia virus.

Subcutaneous inoculation may be made either into the abdominal wall, or into the loose tissue about the flank or at the back of the neck. The hair is clipped, the skin is sterilised with iodine and then pinched up, and the needle is inserted. The technique is the same as that for the guinea-pig.

Intravenous inoculation is employed when material has to be introduced directly into the circulation. The marginal vein of the ear is the most convenient site. The rabbit may be held by an assistant or placed in a special box so that only its head protrudes. The hair over the vein should be dry-shaved with a sharp razor. The vein may be distended for ease of inoculation either by vigorous rubbing with a piece of cotton-wool or by holding the ear over an electric-light globe, when the heat causes a dilatation of the blood vessels. According to the amount of material to be injected, a suitable syringe furnished with a needle is selected and sterilised by boiling or other means. The operator faces the animal and the ear is held horizontally by means of the left hand. The needle is kept as nearly parallel as possible to the vein and the point inserted towards the head of the animal. When the injection is completed, the needle is withdrawn, and a small piece of cotton-wool placed on the vein, which is then compressed between the thumb and finger.

In *removing samples of blood from the rabbit* a similar procedure is adopted. The ear is shaved, sterilised with alcohol and painted with sterile vaseline. The vein is dilated by the heat of an electric bulb or by gently rubbing with benzol or xylol; an incision is made into it by means of a large triangular needle or a sharp scalpel. The blood is then allowed to drop into a test-tube. When sufficient blood has been removed a small piece of cotton-wool is pressed firmly over the incision in the vein. If xylol has been used the ear is washed with spirit and lightly smeared with vaseline (*vide* p. 131).

Cardiac puncture.—Vide p. 132.

Intraperitoneal inoculation is carried out as in the case of the guinea-pig.

Intracerebral inoculation.—The animal is anaesthetised with ether, the hair over the head shaved, and the skin disinfected with alcohol and tincture of iodine. A short incision is made through the scalp at a point situated 2 mm. lateral to the sagittal suture and 1·5 mm. anterior to the lambdoidal suture. The skull is then perforated with a trephine or a mechanical drill and the needle introduced through the opening. About 0·45 c.c. of material can be inoculated into the occipital lobe of a large rabbit. After injection the needle is rapidly withdrawn, the skin sutured, and the area covered with collodion solution.

Rabbits may also be inoculated in the frontal lobe, at a point situated 2 mm. lateral to the median plane on a line joining the two external canthi of the eyes.

RATS

Care must be exercised in handling these animals as the sharp incisor teeth are capable of inflicting a severe wound. They should be held by the loose tissue at the nape of the neck with a pair of crucible tongs or artery forceps, and the animal is kept taut by pulling on the tail. *Intraperitoneal* and *subcutaneous inoculations* are made in a manner similar to that used for the guinea-pig.

Intravenous inoculation may be made into the vein at the root of the tail. The vein should be dilated by immersing the tail in warm water.

MICE

Subcutaneous inoculation.—An assistant grasps the loose skin at the nape of the neck in one hand and the tail in the other. In this manner the animal is held in a fixed position while the needle is introduced under the skin near the root of the tail. Amounts up to 1 c.c. may be injected.

Intraperitoneal inoculation may be carried out if the animal is similarly held and then turned over. For steadiness, the assistant's arms should rest on the table. The injection is made to one side of the middle line in the lower half of the abdomen and amounts up to 2 c.c. can be introduced.

Intraperitoneal inoculation may also be done without an assistant. The animal is held at the nape of the neck with the left hand, and kept extended by holding the tail with the right hand. The left hand is turned over so that the mouse lies on its back in the upturned palm. The tail is then fixed by the little finger of the left hand. The mouse is now firmly held, and the right hand is free to pick up the syringe and make the injection.

Intravenous inoculation may be made into a vein at the root of the tail if a fine needle be used and the vein dilated by placing the tail of the animal in warm water. The maximum amount which can be injected is 0·5 c.c. for a mouse of 20 grams.

A small cylindrical cage made of perforated zinc, and just large enough to hold the mouse with its tail protruding, is useful for this procedure.

Intracerebral inoculation.—The skin over the head is depilated (*vide* p. 235) under slight ether anaesthesia. The animal is completely anaesthetised for the inoculation and the depilated area sterilised with tincture of iodine. A fine-bore needle attached to a 1 c.c. syringe (as used for intradermal inoculation —*vide* p. 236) is employed and easily penetrates the skull. The site of injection is just posterior and lateral to the vertex and the point of the needle is carried through the skull for ⅛ in. to ¼ in. Approximately 0·05 c.c. of fluid can be injected with safety.

AUTOPSY

All experimental animals, whatever the cause of death, should be examined *post mortem* as a routine. When a virulent organism such as the bacillus of plague or of anthrax has been used, special care must be taken, otherwise the infection may be disseminated, with danger to the operator and other workers.

Details will be given of the procedure in conducting an autopsy in the usual manner, and also the method used when dealing with highly infectious organisms.

As a primary reason for the autopsy is to recover organisms previously injected into the animal, the examination must be conducted with strict aseptic precautions.

Materials required :

A suitable animal board or *table*, on which the carcase can be fixed in the supine position.

Instruments.—Three scalpels ; scissors, ordinary size, four pairs ; mouse-toothed forceps, four pairs ; small bone forceps if the skull is to be opened ; a searing iron—a 4 oz. soldering bolt is suitable for the purpose ; sterile capillary pipettes ; sterile Petri dishes ; sterile test-tubes, and tubes of media.

The knives are sterilised in strong lysol (about 20 per cent.) and then placed in a weaker solution (2 per cent.), and the metal instruments by boiling in a sterilising bath—*e.g.* an enamelled " fish-kettle." When ready for use, the tray of instruments is lifted out of the steriliser and laid on a spread towel which has previously been soaked in 1 : 1000 solution of perchloride of mercury.

It is a useful practice, where cultures have to be made, first to immerse the animal completely in weak lysol solution (3 per cent.) for ten minutes. This not only destroys most of the surface organisms, but prevents the dust in the fur from getting into the air and contaminating other materials. The animal is now fixed to the board, and towels moistened with antiseptic are placed over the head and lower extremities.

The instruments are removed from the steriliser. A long median incision through the skin of the abdomen and chest is now made, and the skin widely dissected, exposing the abdominal and chest muscles. With another set of instruments the peritoneal cavity is opened, and the abdominal wall is reflected to each side. With fresh instruments remove the spleen and place it in a sterile Petri dish. Other organs such as the liver and kidneys may be similarly removed. The ensiform cartilage is now tightly gripped with a pair of strong forceps, and by means of a sterile pair of strong scissors a cut is made on either side of the chest through the costal cartilages. The sternum is raised and pulled towards the head. The heart is now exposed. A sterile capillary pipette, furnished with a teat, is passed through the heart wall. Blood can thus be withdrawn and inoculated into various media. If the autopsy has been properly performed, it is not necessary to sear the surface of the heart. The lungs are then removed with fresh instruments, by cutting each organ free at the hilum. Care must be taken not to open into the oesophagus if the lungs are to be used for cultural purposes.

After the organs to be used for culture have been removed and placed in separate Petri dishes, the autopsy can be completed.

While the instruments are again being boiled the naked-eye appearances of the organs should be studied. For culture the spleen gives the best results, but the other solid viscera may be similarly used. The organ is cut with sterile instruments and a small portion is taken up with a stiff wire and smeared on the surface of solid media. Liquid media are inoculated with a small fragment of the tissue.

In conducting *post-mortem* examinations, various animal diseases, such as worm infestation, coccidiosis, pseudo-tuberculosis, etc., may be noticed, and the worker should be familiar with their appearances.

When the animal is infected with highly pathogenic organisms the worker *must* wear rubber gloves. The animal is soaked in antiseptic solution as before, and nailed to a rough piece of board of the appropriate size. This board is then placed in a large enamelled iron tray. The autopsy is carefully performed in the usual way. The carcase is finally covered with 10 per cent. lysol, which flows over the board and into the tray. The whole contents of the tray—board and carcase—are then destroyed in a furnace. The rubber gloves, instruments and tray are thoroughly sterilised.

When performing animal autopsies we strongly advise the wearing of a large overall made of waterproof material, and, in addition, the use of some form of glasses or goggles to protect the eyes.

CARE OF ANIMALS [1]

General Directions

Litter for all animals may be sawdust, wood chips or peat moss.

All cages and runs should be cleaned out twice weekly. The daily duties of the animal-keeper should be written out and posted up in the animal-house. Feeding-troughs should be of earthenware, about four inches in diameter. They must be cleaned daily, and in the case of experimental animals it is important that the troughs are returned to their respective cages.

Separate barrows should be used for food stuffs and soiled bedding. The runs and cages should be regularly inspected for sick animals which should be removed and kept separate. It is very important that the temperature of the animal house be kept as even as possible. Draughts should be avoided. Guinea-pigs particularly are susceptible to changes of temperature, and deaths are frequent amongst inoculated animals if the temperature is allowed to vary.

GUINEA-PIGS

Guinea-pigs should be fed twice daily.

Morning—	
Oats, 1 part	1 oz. per animal
Bran, 3 parts	
Water (for stock in runs)	
Afternoon—	
Hay	2 oz.
Cabbage, kale or other green food supplemented by roots	2 oz.
Water as above	

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[1] For full details of the care of laboratory animals, reference should be made to *A System of Bacteriology*, Medical Research Council, 1931, vol. ix., chap. 17, from which certain of the data in this section are quoted by permission of the Controller, H.M. Stationery Office.

Cages.—Stock runs should be about 3 ft. square, with sides 1 ft. 8 in. high.

For experimental animals, galvanised-iron cages are best, as they are more easily cleaned and sterilised after use. A convenient size is 13½ × 8½ × 6½ in., fitting in a loose tray 14½ × 9½ × 1¾ in.

Breeding.—Period of gestation seventy days ; animals may be used for breeding after six months ; three litters yearly, average litter three ; young weaned at fourteen days.

Common Diseases

Pseudo-tuberculosis (*vide* p. 484).—May be either acute or, more commonly, chronic. In the acute type the animal dies in a few days. In the chronic type the liver, spleen and mesenteric glands show very numerous yellowish-white areas scattered through them, somewhat suggestive of tuberculosis. Often a whole stock becomes infected, and experimental animals frequently die before the experiment is completed.

Respiratory Tract infections.—Guinea-pigs are liable to pneumonia and pleurisy, often with haemorrhage and septicaemia due to such organisms as the pneumococcus, pneumobacillus, *Pasteurella* group, etc..

Intestinal infections.—Organisms of the *Salmonella* group, e.g. *B. aertrycke*, are often responsible, and epizootics are liable to occur in overcrowded stock.

Virus diseases, such as guinea-pig paralysis, may be met with.

RABBITS

Feeding, per animal—

Morning—	
Oats, 1 part	2½ oz.
Bran, 3 parts	
Water	
Afternoon—	
Hay	4 oz.
Cabbage, kale or other green food supplemented by roots	3 oz.
Water	

Cages.—Galvanised iron or zinc should be used. A convenient size is 19½ × 14 × 14 in., with a door in front, resting in a loose tray 20½ × 15 × 2 in. Young rabbits up to three months of age may be housed together, after that time the sexes should be separated.

Breeding.—Period of gestation thirty-one days; animals may be used for breeding after six months; four litters yearly; average litter four; young weaned at six weeks. The mother should be disturbed as little as possible for the first ten days after parturition.

Common Diseases

Pseudo-tuberculosis (*vide* p. 484).—This is a chronic disease, in which the animal loses weight and eventually dies. The liver and spleen particularly show numerous well-defined yellow areas like miliary tubercles.

Respiratory Tract infections—of which *Snuffles*, due to *B. lepisepticus*, is the most common. Infected animals should be destroyed at once.

Intestinal infections.—Diarrhoea may be due to organisms of the *Salmonella* group—e.g. *B. aertrycke.* It is also sometimes caused by *coccidiosis.*

Parasitic Mange.—This usually affects the ears, and is best treated by liquid paraffin containing 1 per cent. of phenol.

Worms. — The cysticercus stage of the dog tape-worm, *Taenia pisiformis*, is the commonest type of infestation, and is characterised by numerous cysts in the omentum, and occasionally in the liver.

Ulcerative lesions of the genitals due to *Treponema cuniculi* (which is very similar morphologically to *Treponema pallidum*, *q.v.*) are frequently seen.

RATS

Feed only once daily, preferably in the afternoon. Give mash of boiled meal, mixed with fresh oats and bran and made into balls the size of a walnut. An adult rat requires 2 oz. of this daily. Also give fresh cabbage in the mixture three times weekly, and some biscuit and cod-liver oil once a week. Rat food should be given immediately it is made, and not allowed to remain over till the next day.

It is more convenient to use "rat feeding cubes"; they are sold commercially[1] and consist of a balanced mixture of ground cereals, meat, fish and milk-proteins, with yeast and cod liver oil. Three cubes per day are provided for each adult animal.

Drinking water from a special glass container should always be available.

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[1] Obtainable from North-Eastern Agricultural Co-operative Society Ltd., Bannermill Place, Aberdeen.

Cages.—Galvanised-iron cages with narrow mesh are used for experimental animals.

For stock animals large wooden boxes with zinc bottoms and perforated zinc tops, 36 × 18 × 15 in., are suitable.

Breeding.—Period of gestation three weeks; three to four litters yearly; average litter six; young are weaned at six weeks, and the female rested a further two weeks.

Common Diseases

Rats suffer rarely from intercurrent infections, except mange.

Respiratory Tract infections—e.g. pneumonia—may occur, but not frequently.

Intestinal infections.—*Salmonella* infections—e.g. ***B. enteritidis***—may cause fatal epizootics.

Mange appears especially at the root of the tail and ears, as a grey warty condition. The animals should be examined twice weekly, and if infected the parts should be smeared with an ointment composed of flowers of sulphur, 2 parts; sodium carbonate (anhyd.), 1 part; lard or vaseline, 16 parts.

MICE

Feeding.—Feed only *once* daily, preferably in the afternoon, with stale dry bread soaked in water, and squeezed and made up in pieces the size of a walnut, or alternatively with a mixture of oats 1 part, and bran 3 parts, moistened and made up into small balls as above; allow one per animal. A little cod-liver oil should be placed in the mash once weekly. A pinch of canary seed and millet seed should be allowed each animal weekly.

Alternatively one "rat feeding cube" (p. 245) per day for each adult mouse provides a completely balanced diet. Drinking water is essential and should be provided from a special glass container.

Cages.—Stock cages for breeding, etc., are made of wood with a zinc bottom. The lid is of perforated zinc with wooden edges. The size is 24 × 12 × 12 in.

For individual mice in a one- or two-day experiment—*e.g.* pneumococcus inoculation—a 1 lb. or 2 lb. screw-capped preserving jar, with a piece of perforated zinc in place of the glass portion of the lid, is most convenient.

Breeding.—Mice are easily bred. Place one male and two to five females in the box. Remove male after fourteen days and the females will litter together. Period of gestation eighteen to twenty-one days; four litters yearly; average litter four Young are weaned at one month; female is rested a further two weeks. It is advisable to replace breeding stock after three litters. An increase of millet and canary seed should be given to breeding animals.

Common Diseases

Intestinal infections due to organisms of the *Salmonella* group (*B. enteritidis* and *B. aertrycke*), and termed " Mouse typhoid," may produce severe epizootics. Existing stock should be destroyed, boxes disinfected and fresh stock obtained.

Infectious ectromelia.—A virus disease occurring in either an acute or chronic form. In the acute disease there is necrosis in the liver and spleen. In the chronic form there is enlargement of one foot (usually hind), due to oedema, followed by an exudation of serous fluid and scab formation, after which gangrene either of a digit or whole foot may supervene.

Epizootic infection by *Streptobacillus moniliformis* (*vide* p. 502) may sometimes occur in mouse stocks.

Ringworm is met with, and also favus (p. 569).

Worms may occasionally cause ill-health or death.

Tumours are not uncommon, particularly mammary carcinoma.

CHAPTER VIII

IMMUNOLOGICAL AND SEROLOGICAL METHODS AS APPLIED TO BACTERIOLOGY[1]

THE WIDAL REACTION; AND OTHER AGGLUTINATION TESTS

THE nature of the *Widal agglutination reaction* and its applications in the diagnosis of enteric fever are referred to on pp. 28, 37, 440.

The underlying principle of the technique of agglutination tests is to examine serum *quantitatively* for agglutinins towards the particular organism. For this purpose the most reliable method, and that usually adopted, is to mix varying dilutions of serum with a fixed quantity of a uniform and stable suspension of the organism (made up in saline solution), the mixtures being placed in narrow tubes, kept at 37° C. or 50°–55° C. for a certain length of time, and then examined for *visible* agglutination or flocculation of the suspension. The agglutinated organisms sediment rapidly, and the reaction can also be gauged by the amount of deposit in the tubes and the clarity of the supernatant fluid. The strength of the reaction can be stated in terms of the highest dilution (" titre ") which produces agglutination.

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[1] Only those immunological and serological methods which are applicable to routine bacteriological work are dealt with. For other methods, and the preparation and testing of therapeutic antisera, reference should be made to larger works on bacteriology and immunity.

DIRECTIONS FOR OBTAINING SPECIMENS OF BLOOD SERUM FOR THE TEST

The blood specimen should be taken by vein puncture [1] (*vide* p. 182), so as to obtain a satisfactory amount of serum for quantitative examination with different enteric group organisms, as is generally required in carrying out this test. At least 5 c.c. of blood should be obtained, and the blood immediately transferred from the syringe to a stoppered sterile tube or screw-capped bottle and allowed to clot. When the serum has separated, it is pipetted off into a sterile tube.

A small amount of serum may be obtained by the following method.

The *requisites* are : triangular needle, small glass-cutting file, spirit lamp or Bunsen burner, gauze or cotton-wool, methylated spirit, and a Wright's blood capsule (1½–2 in. of glass "quill" tube, 5 mm. bore, drawn out at the ends to capillary dimensions and sealed, with one of the capillary limbs bent in a semicircle).

The blood is drawn by puncturing the skin of the finger near the base of the nail. Sterilise the needle and the patient's finger with spirit. Break off the tips of both ends of the capsule, notching first with the file. Puncture the skin by a deep thrust of the needle. When a large drop of blood collects, dip the end of the curved limb of the capsule in it and allow the blood to pass into the capsule by capillary action. The capsule should, if possible, be filled three-quarters full. If blood does not flow freely, pressure may be applied by winding a narrow bandage round the base of the finger. A second puncture may facilitate the filling of the capsule. The success of the method depends on obtaining a *continuous* free flow of blood. If there is any delay and the blood coagulates in the capillary part of the capsule before the required amount is collected, another capsule must be used. To close the capsule, heat the straight end in the flame and seal off the tip. As this end cools, the blood is retracted from the bent limb, which may then be sealed without heating or charring the blood. Both ends must be *completely* sealed.

After the blood coagulates in the capsule, the serum can be separated by centrifuging, the bent limb serving to hang the capsule on the rim of the centrifuge tube. A hand centrifuge is sufficient for this purpose (*vide* p. 289). The capsule is opened by filing and breaking it at the bent end, and the serum is drawn off by means of a capillary pipette (*vide* p. 164).

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[1] In general medical practice the Behring Venule is a most convenient instrument for obtaining a blood sample from a vein (*vide* p. 187).

Some workers prefer a blood capsule of smaller calibre and with both capillary ends straight.

THE AGGLUTINATION TEST

In the routine Widal reaction the patient's serum is tested simultaneously with each of the organisms likely to be responsible for enteric fever in the particular region, *e.g.* in this country at the present time, *B. typhosus* and *B. paratyphosus B.* In other parts of the world *B. paratyphosus A* or *C* may require to be included. It will be shown later how additional diagnostic information may be obtained by testing separately for H and O agglutinins (*vide* p. 257). Thus, the Widal test generally involves two or more parallel tests with different enteric group organisms, and also different forms of the same organisms. *To simplify description a single test will be referred to.*

Requisites :

1 c.c. pipette graduated to the tip in 1/10ths and 1/100ths ; 0·1 c.c. pipette graduated to the tip in 1/100ths and 1/500ths ; a rubber teat, or preferably a mouth-piece for pipetting by suction—*i.e.* 3 in. of 5 mm. bore quill tube with 9–12 in. of rubber tubing attached which can be fitted to the top of the pipette (*vide infra*). The free end of the mouth-piece is "smoothed" in the Bunsen flame.

Steriliser with boiling distilled water for pipettes, etc. (*vide* p. 82) ; sterile 0·85 per cent. saline ; test-tubes 3 × ½ in. ; agglutination tubes 3 × ⅙ in., or Dreyer's agglutination tubes the lower ends of which are conical so that the amount of sediment can easily be estimated ; test-tube racks suitable for the tubes used ; small beaker or conical test-glass for saline solution ; grease pencil for marking tubes ; capillary pipette (*vide* p. 164).

Bacterial Suspension

The strain used must be carefully selected and known to be suitable for the diagnostic agglutination test. It must be a motile "smooth" form of the particular type, and if the organism is "diphasic" (*vide* p. 42), e.g. *B. paratyphosus B*, must represent the specific phase.

(1) It has become a common practice in this country to use standard suspensions such as those described later (p. 257).

(2) Should it be desired to prepare a small quantity of suspension for immediate use the following method can be adopted : add in fractions 5 c.c. of physiological saline to a well-grown twenty-four hours' agar slope culture, and emulsify the growth with the aid of a platinum loop. This suspension can be standardised to a suitable opacity, *e.g.* tube 5, Brown's opacity standards (*vide* p. 286). The suspension is decanted and allowed to stand for half-an-hour until bacterial clumps and fragments of agar have sedimented. Alternatively, it may be centrifuged for one minute.

Serum Dilutions

First make up a 1 in 15 dilution of the patient's serum, and from this prepare a series of doubling dilutions in small (3 × $\frac{1}{2}$ in.) test-tubes :

①	②	③	④	⑤	⑥	⑦
1 in 15	1 in 30	1 in 60	1 in 120	1 in 240	1 in 480	CONTROL—NO SERUM

The actual procedure is as follows.

In the rack place seven small (3 × $\frac{1}{2}$ in.) tubes as above.

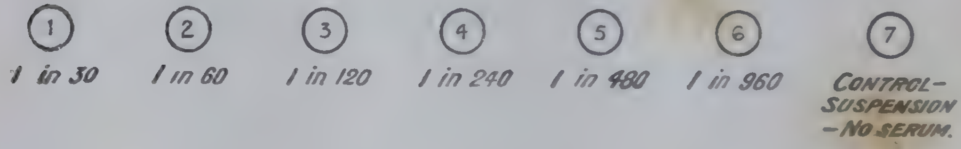
Add 0·4 c.c. saline to each of the tubes ② to ⑦.

In a separate tube add 0·1 c.c. patient's serum to 1·4 c.c. saline—*i.e.* a 1 in 15 dilution.

Wash out the pipette thoroughly in saline solution.

Add to tubes ① and ② 0·4 c.c. of the 1 in 15 dilution of patient's serum. The dilution of serum in tube ② is now 1 in 30. Withdraw 0·4 c.c. from tube ② into tube ③, making now in tube ③ a 1 in 60 dilution of serum. Withdraw 0·4 c.c. from tube ③ into tube ④—*i.e.* 1 in 120—and so on till a dilution of 1 in 480 is obtained in tube ⑥. Withdraw and discard 0·4 c.c. from tube ⑥. Tube ⑦ contains saline only.

0·4 c.c. of the bacterial suspension is added to each tube and the pipette is then sterilised. The dilutions of serum are now :



The mixtures are transferred with a capillary pipette to agglutination tubes, starting with tube ⑦.

To observe agglutination of the H type it is usually sufficient to incubate at 37° C. for one and a half hours and then leave for half-an-hour at room temperature. Some observers prefer to incubate the tubes in a water-bath at 50°–55° C. for two hours. " Large-flake " clumping or agglutination can easily be detected with the naked eye in a satisfactory light. The flocculi also tend to sediment, and the deposit is quite perceptible in the narrow tubes.

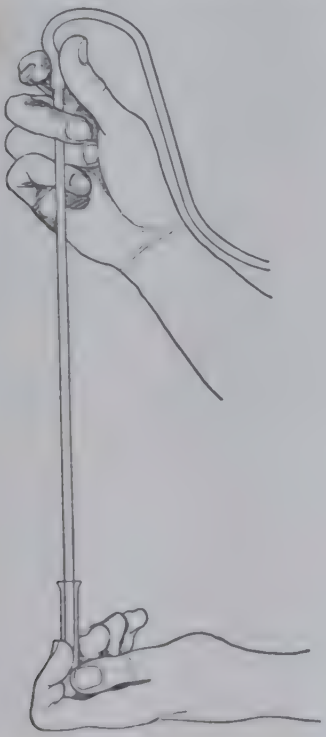
When agglutination of the O type is tested, readings should be made after four and twenty-four hours, as this form of reaction develops slowly (*vide* p. 41). It is also advisable to incubate at 50°–55° C.. The clumps are small and " granular " and observations are aided by the use of a hand-lens and a strong illuminant.

If less than 0·1 c.c. of serum is available, the test quantity of diluted serum and bacillary suspension must be adjusted accordingly.

Further references to the Widal reaction are made on pp. 255, 440.

METHOD OF PIPETTING WITH A GRADUATED PIPETTE AND MOUTH-PIECE

The glass mouth-piece is held between the teeth at the right corner of the mouth, and the top of the pipette is supported between the second and third fingers of the right hand so that the rubber tube immediately above the end of the pipette can be compressed between the thumb and the first finger (see figure). The fluid is drawn up (*e.g.* from a test-tube) into the pipette by suction until the column extends just above the required graduation mark. The end of the mouth-piece is then closed by the tongue, and the column of fluid is depressed to the particular level by gentle pressure on the rubber tubing between the thumb and forefinger. *With the tongue still firmly applied to the mouth-piece* this exact volume of fluid can be transferred from the original tube and then expelled from the pipette into another tube.


]

This method, for which the necessary skill is soon acquired by practice, permits of *accurate* and *rapid* measurements of even small volumes.

The glass-tube of the mouth-piece can be sterilised by flaming.

Measurement of Serum and other Fluids by drops.—Some serological workers prefer to make measurements of serum, saline, etc., in terms of drops delivered from a suitable dropping pipette. This consists in its simplest form of a piece of quill tube drawn out to capillary dimensions (as in the capillary pipette, *vide* p. 164). Alternatively, special dropping pipettes can be purchased. The pipette is actuated by a teat. When used it is held vertically and the fluid in it is allowed to drop slowly from the capillary stem.

The following exemplifies the carrying out of an agglutination test by this method. In a suitable rack place a row of five Dreyer's agglutination tubes (*vide* p. 250) and a test-tube (about $3 \times \frac{3}{8}$ in.) which may be called the "dilution tube." Into this tube measure with the dropping pipette 18 drops of normal saline. Similarly add 2 drops of the serum and mix. This yields a 1 in 10 serum-dilution. The pipette is then thoroughly washed out with saline. Saline, serum-dilution and bacterial suspensions are now added to the five agglutination tubes as follows :

Tube	1	2	3	4	5
	Drops				
Saline . . .	0	5	8	9	10
Serum, 1 in 10 . .	10	5	2	1	0
Bacterial suspension .	15	15	15	15	15
Final dilution of serum	1 in 25	1 in 50	1 in 125	1 in 250	

Tube 5 constitutes the control. The tubes are incubated and the observations then made (*vide* p. 252).

For full details of the preparation and use of dropping pipettes see *A System of Bacteriology*, Medical Research Council, London, 1931, vol. ix., chap. 14.

Slide Agglutination.—This method is useful where only small quantities of culture are available, as in the diagnosis of whooping-cough (*vide* p. 501), or where agglutination is carried out with undiluted serum, *e.g.* in typing pneumococci or typing streptococci by Griffith's method, and it is necessary to use as small a quantity as possible. The method may be applied likewise for typing organisms of the *Salmonella* and dysentery groups. Slide agglutination is only practicable when the clumping of organisms occurs within a minute or so; it is not suitable where the mixture of organisms and serum has to be incubated.

The procedure can be carried out quite readily on an ordinary slide, but where a number of agglutination tests have to be made it is more convenient to use a piece of ¼-in. polished plate glass about 6 in. × 2 in. A long horizontal line is ruled with a diamond through the middle of the glass from end to end, and then a number of lines are ruled at ½-in. intervals at right angles to this line, thereby dividing the glass into a series of divisions.

A drop of saline is placed in one of the divisions, and a small amount of culture from a solid medium emulsified in it by means of a platinum wire. It is then examined through a hand lens (8 or 10 ×), or the low power of a microscope (p. 170), to ascertain that the suspension is even, and the bacteria are well separated and not in visible clumps. With a small loop, 1½ mm. diameter, made from thin platinum wire (about 32 gauge) take up a drop of the serum and place it on the slide just beside the bacterial emulsion. Mix the serum and bacterial suspension and examine with the hand lens, or place on the stage of the microscope. Agglutination when it occurs is rapid and the clumps can be seen with the naked eye, but the use of some form of magnification is an advantage. For control purposes, two drops of saline can be placed in adjacent divisions and bacterial culture emulsified in both, one only being mixed with the serum. With streptococci a broth culture is used, and in this case the growth has usually sedimented at the bottom of the tube. The supernatant fluid is pipetted off and the residue of the fluid and the organisms are mixed with the pipette. Two drops of this fluid are placed on the slide and a small loopful of the serum mixed with one of them and examined as described above.

After the test, the glass slide is wiped with a pledget of cotton-wool soaked in 3 per cent. lysol solution, washed under the tap and dried.

DREYER'S STANDARD AGGLUTINATION METHOD

It is shown in Chapter XV how certain organisms—e.g. *B. typhosus*—are susceptible to agglutination by normal serum, which has to be allowed for in interpreting results of diagnostic agglutination tests. Owing to the fact also that cultures of a particular organism may vary in their agglutinability, a method has been adopted for standardising these tests (Dreyer). Suspensions of the particular organisms are prepared in bulk and treated with formalin (for H agglutination) or alcohol (for O agglutination)—*vide infra.* Such suspensions remain suitable for agglutination tests over long periods. A newly prepared suspension is compared with a standard suspension as regards its agglutinability by a particular antiserum, and any difference noted is stated as a factor by which the titres of sera tested with the new suspension can be corrected so as to render the results comparable with those given by the standard suspension. The bacterial suspensions can be obtained from the Standards Laboratory, School of Pathology, Oxford University.

The method of the Standards Laboratory for carrying out an agglutination test and recording results is as follows.

The measurements may be made either by the dropping method (*vide supra*) or with graduated pipettes. We recommend the latter, the technique being as described on p. 253.

Set out in a rack seven Dreyer's agglutination tubes and in two small test-tubes (about 3 × ½ in.) make up 1 in 10 and 1 in 100 dilutions respectively of the serum. Then add saline, serum-dilution and bacterial suspension to the agglutination tubes as follows :—

Tube	1	2	3	4	5	6	Control
c.c. saline .	0	0·2	0·3	0	0·2	0·3	0·4
	1 in 10			1 in 100			
c.c. serum-dilution .	0·4	0·2	0·1	0·4	0·2	0·1	0
c.c. bacterial suspension .	0·6	0·6	0·6	0·6	0·6	0·6	0·6
Final dilution of serum :	1 in 25	1 in 50	1 in 100	1 in 250	1 in 500	1 in 1000	

The tubes are incubated in a water-bath at 50°-55° C., one-third only of the column of fluid in the tubes being under the water level. Incubation is continued for two hours for H agglutination and eighteen to twenty-four hours for O agglutination, followed by fifteen to twenty minutes at room temperature. The agglutination is observed (with the naked eye) by artificial light against a dark background. Well-marked agglutination without sedimentation is designated "standard agglutination." ("Standard agglutination tubes" are supplied by the Standards Laboratory to show the precise degree of clumping known as "standard agglutination.") If standard agglutination occurs with the bacterial suspension up to a certain dilution of serum and the label of the bottle of suspension states the agglutinability to be standard, the "standard titre" of the serum is the dilution referred to. If the agglutinability is "standard × " a given factor, the standard titre is found by dividing the dilution-denominator by the factor. Thus, if standard agglutination occurs in a 1 in 200 dilution and the given factor is 0·5, then standard titre would be 200/0·5, *i.e.* 400. The diagnostic interpretation of results is dealt with later in connection with enteric fever (p. 440) and other infections.

These methods are fully described in the *Medical Research Council's Special Report Series*, No. 51, and in a pamphlet of directions issued by the Standards Laboratory. See also *A System of Bacteriology*, Medical Research Council, London, 1931, vol. ix., chap. 14, pp. 186–189.

BACTERIAL SUSPENSIONS FOR TESTING H AND O AGGLUTININS[1]

Formolised suspensions of motile flagellate organisms, e.g. *B. typhosus*, show the "large-flake" agglutination characteristic of the H antigen (*vide* p. 41). These are prepared by adding 0·1 per cent. of formalin to a twenty-four hours' veal extract broth culture or by suspending an agar culture in saline containing 0·1 per cent. formalin. Formalin interferes with O agglutination, and if the agglutinin formed in a case of enteric fever is mainly of the O type a formolised suspension would fail to detect it.

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[1] See Felix, A., *Lancet*, 1930, i., 505.

The reactivity of the H antigen can be annulled by alcohol, and if cultures are treated with alcohol a standard suspension representing the O antigen alone can be obtained (*vide infra*).

Formolised and alcoholised suspensions supply, therefore, the necessary reagents for testing H and O agglutinins respectively.

It is also advisable to use for such tests selected strains which are sensitive to H and O agglutination respectively.[1]

Method of making O-Agglutinable Suspensions.—Plate out the organism and select a smooth colony. Subculture this on phenol-agar (1 in 800 phenol). Scrape off the growth in the minimum amount of saline, emulsifying very carefully, and add about 20 times the volume of absolute alcohol. Heat at 40°–50° C. for half-an-hour. Centrifuge (if necessary) and suspend the deposit in saline to the proper density, with chloroform as a preservative. This emulsion keeps moderately well, but if an old suspension is used, it should be centrifuged and re-suspended in fresh saline. The original practice of keeping O suspensions in alcohol and diluting when ready for use is not recommended as the alcohol eventually annuls the agglutinability of the organisms.

OTHER AGGLUTINATION TESTS

The agglutination technique described above is also applicable to diagnostic tests with *B. melitensis*, *B. abortus*, *B. proteus* X19 (Weil-Felix reaction of typhus fever), etc.. The series of dilutions tested can, of course. be varied according to the range within which agglutination is likely to occur (*vide* pp. 467, 489, 561). It is essential in all cases to make these tests quantitative so that the "titre," or highest dilution in which agglutination occurs, can be estimated.

AGGLUTINATION TESTS USED FOR THE SEROLOGICAL IDENTIFICATION OF CERTAIN ORGANISMS BY MEANS OF SPECIFIC ANTISERA

These tests are carried out by a similar technique. In this case the series of dilutions depends on the titre of the serum for the homologous organism. Thus if the titre were 1 in 16,000, the following range of dilutions might be tested: 1 in 1000 *to* 1 in 32,000 in a series of doubling dilutions. In general for identification of an unknown organism it should agglutinate in approximately as high a serum-dilution as a known homologous organism.

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[1] See Felix (footnote, p. 257).

If the organism is a motile species and it is desired to identify both H and O antigens, formolised and alcoholised suspensions respectively, are tested with H and O agglutinating antisera (*vide infra*). It should be noted that in the *Salmonella* group the H antigen may occur in either a specific or non-specific phase. The serological identification of these organisms is considered more fully on p. 448.

Special applications of the agglutination technique —*e.g.* in the identification of serological types—are referred to in later chapters.

PREPARATION OF AGGLUTINATING ANTISERA

The instructions given here apply particularly to organisms of the typhoid-paratyphoid group.

Rabbits are used for immunisation, and large healthy animals should be selected, not under 2000 grams in weight.

The purity and identity of the culture used should be carefully ascertained beforehand, and in view of variability in antigenic composition the culture selected should be such that it represents the motile "smooth" form and the specific phase of the particular species (*vide* pp. 42, 449).

The rabbits are injected intravenously (*vide* p. 238) at intervals of five to seven days with a suspension in saline of a twenty-four hours' slope culture killed by exposure for one hour at 60° C.. The following series of doses may be given: 1/20, 1/10, 1/5, 1/3 and 1/2 culture. These doses are easily measured by emulsifying a slope culture in a given volume of saline and then injecting the appropriate fraction.

In the case of organisms of high toxicity—e.g. *B. dysenteriae* (Shiga)—it is necessary to start with even lower doses—*e.g.* 1/100 of a culture.

With certain species—e.g. *B. aertrycke*—higher titres may be obtained if living organisms are injected. To commence with, very small amounts—*e.g.* 0·01 c.c.—of a young living broth culture should be injected intravenously. As the animal becomes immune larger doses may be given, until several c.c. of the living culture can be tolerated.

Other methods for standardising dosage may be used—*e.g.* where the doses are stated in terms of the number of organisms, as in the administration of vaccines (*vide* p. 285)—but the system indicated above is simple and sufficiently accurate for ordinary purposes.

When separate H and O agglutinating antisera are required for motile bacteria, immunisation is best carried out with selected strains known to be suitable for the purpose. For the production of the O agglutinin an alcoholised culture may be used as the antigen. A non-motile variant also serves well as a pure O antigen. In making tests with H and O antisera, culture-suspensions are formolised for H agglutination and alcoholised for O agglutination.

Seven to ten days after the last injection a specimen of blood is withdrawn from an ear vein (*vide* p. 238), and the serum is tested for its agglutinating power towards the strain used for immunisation. A series of dilutions is tested, and if agglutination occurs in a 1 in 1600 or higher dilution,[1] the animal is bled from the neck vessels or by cardiac puncture (*vide* p. 132), the blood is allowed to coagulate in a sterile stoppered measuring cylinder, placed overnight in the refrigerator, and the serum is then separated. 0·1 c.c. of a 5 per cent. solution of phenol in physiological salt solution is added for each c.c. of the serum—equivalent to 0·5 per cent. pure phenol. This prevents bacterial growth resulting from any accidental contamination. (Glycerol may also be used as a preservative, an equal volume being added to the serum, or 0·1 per cent. *p*-chlor-*m*-cresol.) The serum may be stored in 1 c.c. or 5 c.c. stoppered bottles, or ampoules may be used. Alternatively it may be kept in sterile quill tubes, about 1 c.c. in capacity, drawn out at both ends to capillary dimensions. The tubes are filled by suction, applying the mouth-piece used in pipetting (*vide* p. 253), and the ends are sealed in the Bunsen flame. The serum should be kept in the refrigerator (about 4° C.) and will retain its potency for long periods (three years). The temperature should not be allowed to fall below 0° C., as the solidification of the serum by freezing may be deleterious owing to the separation out of the phenol in the pure state.

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[1] More powerful agglutinating sera may of course be obtained: in immunising animals, 1 in 1600 is merely the *minimum* titre which should be aimed at.

Antisera can be preserved in the dry state by the methods described on p. 267 for the preservation of complement. The potency of antisera is retained over a considerably longer period when dried than when stored in fluid form.

AGGLUTININ-ABSORPTION TESTS

Agglutinins, like other antibodies, combine firmly with their homologous antigens, and by treating an agglutinating antiserum with the homologous bacteria and then separating the organisms by centrifuging, it is found that the agglutinin has been "absorbed" or removed by the organisms from the serum.

In certain cases, to prove the serological identity of an unknown strain with a particular species, it may be necessary to show not only that it is agglutinated by a specific antiserum to approximately its titre but also that it can absorb from the serum the agglutinins for the known organism. This becomes necessary owing to the fact that, on immunising an animal with a particular bacterium, "group antibodies" for allied organisms are developed, and in some cases these may act in relatively high titre. "Absorption" with a heterologous strain would only remove the group agglutinins without affecting the specific agglutinin. These effects are exemplified in the *Salmonella* group (*vide* p. 416). The general method of carrying out such absorption tests is to mix a dense suspension of the organism—*e.g.* twenty-four hours' growth on a 4 in. plate of nutrient agar, suspended in 1 c.c. saline and killed at 60° C. (thirty minutes)—with an equal volume of a suitable dilution of the serum—*e.g.* sixty-four times the concentration of the known titre. Thus, if the titre is 1 in 1600, the dilution used would be 1 in 25. The mixture is incubated for three to four hours at 37° C. and the serum is then separated from the bacteria in a high-speed centrifuge. (In some cases for complete absorption the process may require to be repeated with a similar fresh quantity of bacteria.) The dilution of the serum would now be approximately double the original dilution—in the example taken (*vide supra*) 1 in 50. From the treated serum a series of doubling dilutions is prepared as in direct agglutination tests (*vide* p. 251), so that, when an equal volume of bacterial suspension is added, the series will reach to the known titre of the serum. In the example taken above, the following series of dilutions would be prepared :

1 in 50 1 in 100 1 in 200 1 in 400 1 in 800,

and after the addition of bacterial suspension these would become

1 in 100 1 in 200 1 in 400 1 in 800 1 in 1600.

A control tube is also included, containing suspension but no serum, and the general technique is that employed in direct agglutination tests.

Thus, the identity or non-identity of an unknown culture (*X*) with a known (*A*) may be investigated by agglutinin-absorption as follows :

I. Absorb, as above, antiserum to *A* with a dense suspension of organism *X*=*X*-absorbed serum.

II. Test the agglutinating power of *X*-absorbed serum for *A* and *X*.

(A control test would show that the antiserum to *A* after absorption with *A* agglutinates neither organism.)

Results :

(*a*) The absorbed serum agglutinates neither *A* nor *X*. Conclusion—the organisms are apparently identical ; to establish this conclusion completely an antiserum to *X* after absorption with *A* should agglutinate neither organism.

(*b*) The absorbed serum fails to agglutinate *X*, but still agglutinates *A*. Conclusion—the organisms are not identical.

Absorption tests in their application to antigenic analysis are referred to on p. 446, and in carrying out such tests attention must be paid to the condition of the culture used, both as regards (1) the somatic antigen (state of the organisms, whether "smooth" or "rough"), and (2) the flagellar antigen (the phase of the organisms, whether "specific" or "group").

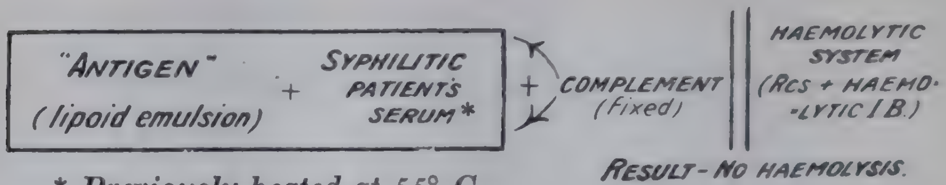
WASSERMANN SYPHILIS REACTION

This reaction depends on the "fixation" of complement by an emulsion of certain lipoid substances (phosphatides, such as lecithin) along with the *heated* serum of a person infected with syphilis, and constitutes an important diagnostic test.

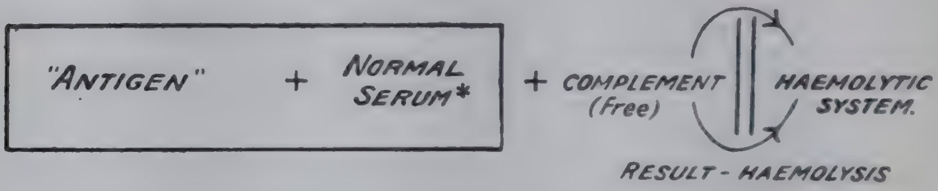
The effect is not a true immunity reaction, though the lipoid emulsion *plus* syphilitic serum fixes complement in the same way as a bacterial or other antigen *plus* its homologous antiserum (*vide* p. 43). Possibly the lipoid plays the part of a hapten in this reaction. Though some observers attribute the phenomenon to a physico-chemical change in the serum, the evidence suggests that it depends on the presence in the syphilitic serum of a "lipoidophile" antibody-like principle which is stable at 55° C..

For complement-fixation tests, an indicator of the presence of complement is required. The "haemolytic system" used in these tests serves this purpose (*vide* p. 39). It consists of the red corpuscles of a particular animal species "sensitised" with the corresponding haemolytic immune body (I.B.)—*e.g.* the red cells of the ox or sheep *plus* the serum of a rabbit which has been immunised with the red cells of the species used. The immune body in the serum is thermostable. The serum is heated at 55° C. to annul the natural complement, and stored in bottles or tubes or preserved in the dry state (*vide infra*). The heated serum is non-haemolytic by itself, but in the presence of a suitable complement produces lysis of the homologous red corpuscles. Fixation of complement is denoted by the absence of lysis in the haemolytic system.

In its simplest form the Wassermann reaction can be represented as follows :—


"ANTIGEN"
(lipoid emulsion)
+
SYPHILITIC PATIENT'S SERUM*
+ COMPLEMENT
(Fixed)
HAEMOLYTIC SYSTEM
(RCS + HAEMOLYTIC I.B.)
RESULT - NO HAEMOLYSIS.

* *Previously heated at 55° C.*


"ANTIGEN"
+
NORMAL SERUM*
+ COMPLEMENT
(Free)
HAEMOLYTIC SYSTEM.
RESULT - HAEMOLYSIS

It should be specially noted that the Wassermann test is both qualitative and quantitative. Not only does it indicate whether the reaction is positive, but the various degrees of strength of the reaction may also be determined, from a strong positive (+++) to a weak positive (+) or a doubtful positive (±). This quantitative testing is most important in assessing the value of treatment or the completeness of cure.

The technical application of the reaction demands a very accurate standardisation of each reagent. Further, the amount of complement used in relation to the quantities of antigen and serum must be adjusted with such delicacy that the weakest reactions can be accepted as significant.

Although the essential principles of the test and its reagents are the same, several modifications of the technique are employed. The majority of workers use constant amounts of antigen and of patient's serum, with varying amounts of complement. In some cases, constant amounts of antigen and of complement with varying amounts of patient's serum are employed—*e.g.* the standard method recommended by the Royal

Medico-psychological Association for mental hospital practice.[1]

Examples of two different forms of technique are described: (1) In which the various quantities of the reagents are measured with graduated pipettes and added directly to the tubes in which the test is carried out. (2) In which the reagents in the first place are all diluted to a standard volume, variation in actual amount—*e.g.* of complement—being effected by varying the concentration in the standard volume. Thus only one standard volume requires to be pipetted instead of varying quantities, and moreover the method can be applied to small amounts, but it has the disadvantage that it can become very inaccurate in the hands of other than skilled workers.

The preparation of the reagents is essentially the same in the different methods.

METHOD I.

ANTIGEN

(1) 20 grams of *sheep heart-muscle*, carefully freed from fat and fibrous tissue, are *finely* ground with clean sand in a mortar, and extracted for four days at room temperature with 100 c.c. of 96 per cent. alcohol. In this way lecithin and similar substances are extracted from the tissue. The extract is filtered, and pure cholesterol is dissolved in it to the point of saturation. The cholesterol acts by intensifying the " antigenic " properties of the tissue extract.

For the test, a suspension is prepared by adding 1 part of the alcoholic extract to 12 parts of normal saline solution. In preparing the emulsion the *maximum turbidity* should be obtained by running the extract slowly on to the salt solution in a cylindrical measure (or test-tube), and then mixing slowly by rotation of the cylinder held in a slanting position.

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[1] See Mann, S. A., and Partner, F., *Memorandum on the Wassermann Reaction in Mental Hospital Practice*, London County Council, 1931 (obtainable from King & Son, London).

(2) The following *alternative antigen* is also recommended : *human heart-muscle* from the left ventricle is obtained at a *post-mortem* examination (if possible a case of accidental death) ; it is freed from fat, minced finely, and ground for a minute in a mortar with absolute alcohol (1 gram of heart to 9 c.c. of alcohol) and clean sand. The mixture is shaken in a shaking machine for 1½ hours and then filtered. To 1·5 volumes of this extract is added 1 volume of 1 per cent. cholesterol in absolute alcohol. For use, 1 volume of this mixture is placed in a beaker and 29 volumes of saline are measured into a cylinder, the contents of which are then poured rapidly into the beaker. In the actual test, as shown on p. 271, this antigen suspension can be used in place of that described above.

PATIENT'S SERUM

A specimen of blood is obtained by vein puncture (*vide* p. 183) as for blood culture. The blood is then placed in a sterile stoppered test-tube or screw-capped bottle (*vide* p. 317), and allowed to coagulate. It is advisable to obtain about 5 c.c. of blood. The serum is pipetted off after separation and heated in a water-bath at 55° C. for half-an-hour. Heating *eliminates the fallacy of non-specific fixation effects which may occur with normal unheated sera* plus *the antigen*; it also deprives the serum of its complementing property.

COMPLEMENT

Fresh guinea-pig serum is used. It contains an active haemolytic complement for the red corpuscles of the ox or sheep sensitised with the homologous haemolytic antibody. The blood is obtained twelve to eighteen hours before the test, by severing the large vessels of the neck over a 6-in. funnel, from which the blood is collected in a measuring cylinder; it is allowed to coagulate and stand overnight in the refrigerator. The complement in serum too recently withdrawn is apt to be excessively "fixable," and in consequence is unsuitable for the Wassermann test.

If possible the pooled serum of several guinea-pigs should be used.

It should be noted that complement is unstable and deteriorates on keeping at ordinary temperatures. It is advisable throughout the experiment to keep the guinea-pig serum on ice.

PRESERVATION OF COMPLEMENT

Complement can be preserved by keeping the serum frozen at −15° to −20° C..

A more convenient way of preserving complement is to dry it by Craigie's or Hartley's method. The essential principle of these methods is to dry the guinea-pig serum *in vacuo* over fused calcium chloride or phosphorus pentoxide. The vacuum (obtained by means of a Geryk or Hyvac pump) should be within 1–2 mm. of mercury from perfect. The water vapour arising from the serum is immediately absorbed by the calcium chloride or phosphorus pentoxide, so that the rapid vaporisation causes the serum to freeze solid within ten to twenty minutes. The water vapour still continues to arise from the solid material, so that in a few hours the serum is completely dry. The secret of success is the rapid freezing of the complement and the subsequent drying from the frozen state.

In *Craigie's method*, the guinea-pig serum having separated overnight in the refrigerator is pipetted into a previously cooled thin Petri dish (without a cover). The serum should not be more than 3 mm. deep. The dish is supported on three corks, which act as heat insulators, in a large desiccator in which freshly-fused calcium chloride has been placed. The lid is now put on, and the bulk of the air extracted by means of an ordinary water-pump. The desiccator is connected to the vacuum pump and the vacuum maintained for about twenty minutes. Within this time the serum will have frozen solid. The tap of the desiccator is then turned off, the pump disconnected and the desiccator placed in the refrigerator. Next day the complement will be completely dry. The dry powder can conveniently be stored in a stoppered tube or small screw-capped bottle in a closed jar of fused calcium chloride. To reconstitute the serum add 1 part of dried complement to 15 parts of distilled water.

Hartley's method is essentially the same, except that to the guinea-pig serum are added sodium chloride crystals, 3·4 per cent. The salted serum is pipetted very accurately in amounts of 1 c.c. into round-bottom wide-necked ampoules of about 7 c.c. capacity. The ampoules are arranged in a thin glass dish, around which is packed 2 parts ice and 1 part salt, so that the complement is frozen solid. The dish containing the ampoules is then placed in a desiccator over phosphorus pentoxide or preferably fused calcium chloride, and the exhausting and drying carried out as above, the desiccator being left in the refrigerator for about ten days. When the drying process is complete the ampoules are sealed. To reconstitute the serum the ampoules are opened, and 5 c.c. of distilled water added, which produces a 1 in 5 dilution of complement in normal saline. By this method no weighing out of the dried product is necessary, and preparations have been obtained which have retained their activity for several years.

See also **Appendix**—Rayner's method.

Preservation by Sodium Acetate.—A very simple and most convenient method of preserving complement is to add to the serum an equal volume of a solution of 12 per cent. sodium acetate and 4 per cent. boric acid in distilled water.[1] The serum is kept in sterile screw-capped bottles at (approximately) 4° C.. The full haemolytic activity of the serum and the fixability of the complement in the Wassermann reaction are maintained for at least six months. It should be noted in using this preserved complement that it represents a 1 in 2 dilution of the original serum.

See also **Appendix**—Richardson's method.

HAEMOLYTIC SYSTEM

With guinea-pig complement a haemolytic system consisting of ox or sheep red corpuscles sensitised with the appropriate immune serum is used.

Defibrinated blood is obtained at the abattoir (*vide* p. 126). The required quantity is thoroughly mixed with several volumes of normal saline and then centrifuged to separate the corpuscles, the supernatant fluid being pipetted off. This process has generally been designated " washing " the blood corpuscles and is repeated three or four times. The centrifuged deposit of corpuscles after the final washing is suspended in normal saline to form a 3 per cent. suspension, and five minimum haemolytic doses (M.H.D.) of the immune serum are added.

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[1] Sonnenschein, C., *Ztschr. f. Immun.*, 1930, 67, 512.

Burroughs Wellcome haemolytic serum for *sheep* red corpuscles may conveniently be used in preparing a haemolytic system for the test. This anti-sheep haemolytic serum (from the horse) tends to exert a pronounced agglutinating effect on the homologous corpuscles with rapid sedimentation of the cells. It is advisable therefore to add the serum to the corpuscles just before the haemolytic system is required. A haemolytic system of ox cells sensitised with a rabbit *v.* ox immune body is usually free from marked or rapid haemagglutination.

The method of preparing a HAEMOLYTIC ANTISERUM and of estimating its M.H.D. may be summarised as follows : a rabbit is injected intravenously at seven to ten days' intervals with increasing amounts of washed red cells—*e.g.* 0·5 c.c., 1·0 c.c., 1·5 c.c. of the sediment after washing and centrifuging. Alternatively the animal is injected intraperitoneally with 5 c.c., 10 c.c. and 15 c.c. of the washed red cells at similar intervals. Ten days after the last injection a small quantity of blood is withdrawn from an ear vein ; the serum is separated, and its M.H.D. estimated by testing the haemolytic effect of varying amounts (*e.g.* from 0·001 c.c. *to* 0·005 c.c.) on 1 c.c. of a 3 per cent. suspension of red cells along with an excess of guinea-pig complement (*e.g.* 0·05–0·075 c.c.) (see methods given below). As guinea-pig serum contains natural haemolysins for ox and sheep corpuscles, the complement-containing serum must previously be freed from this antibody by mixing equal volumes of the serum and the appropriate red corpuscles (washed), keeping the mixture at 0° C. for one hour and then separating the serum by centrifuging. If the M.H.D. is 0·002 c.c. or less, the animal is bled from the neck vessels or by cardiac puncture (*vide* p. 132). The blood serum is separated, heated for one hour at 55° C., and stored in sealed quill tubes or phenolised and bottled (*vide* p. 261). Alternatively it may be preserved in the dry state (p. 267).

Should the M.H.D. exceed 0·002 c.c., a further injection of blood may increase the haemolytic potency of the serum.

THE TEST

Apparatus required :

Small test-tubes, 3 × ½ in. ; a rack for the tubes ; 1 c.c. pipette graduated to the tip in 1/10ths and 1/100ths ; 0·1 c.c. pipette graduated in 1/100ths and 1/500ths (*vide* p. 250).

As specimens of complement from different guinea-pigs vary quantitatively in their haemolytic effect, it is necessary first to estimate the M.H.D. of the complement to be used. The M.H.D. is the smallest amount required to produce complete lysis of the given quantity of red cells in the presence of excess of immune body. This usually lies between 0·004 c.c. and 0·01 c.c. of undiluted guinea-pig serum for 0·5 c.c. of the haemolytic system.

To measure amounts less than 0·01 c.c. make a 1 in 10 dilution of the serum, and of the diluted serum take ten times the actual amount required, using the 0·1 c.c. pipette graduated in 1/100ths and 1/500ths.

The following exemplifies the estimation of the M.H.D. of complement :—

Tube . . .	1	2	3	4	5
Haemolytic system . .	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.
Complement (diluted 1 in 10)	0·02 c.c.	0·04 c.c.	0·06 c.c.	0·08 c.c.	0·1 c.c.
Haemolysis . .	Trace	Marked	Just complete	Complete	Complete

The M.H.D. is therefore 0·06 c.c. of a 1 in 10 dilution of complement —*i.e.* 0·006 c.c. undiluted complement.

Tubes 1 and 5 can be omitted from the series unless the complement is unusually active or weak.

The actual Wassermann test is shown in the Table below.

Full Test

	Test proper				*Serum Control*		*Antigen Control*	
Tube .	1	2	3	4	1	2	1	2
Normal saline .	–	–	–	–	0·5 c.c.	0·5 c.c.	–	–
Antigen suspension	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	–	–	0·5 c.c.	0·5 c.c.
Patient's serum .	0·05 c.c.	0·05 c.c.	0·05 c.c.	0·05 c.c.	0·05 c.c.	0·05 c.c.	–	–
Complement	2 MHD	4 MHD	8 MHD	12 MHD	2 MHD	4 MHD	2 MHD	4 MHD

Complement dose Control :—

Tube . . .	1	2	3	4
Saline . . .	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.
Complement (diluted 1 in 10) .	0·04 c.c.	0·06 c.c.	0·08 c.c.	0·1 c.c.

Abridged Test

Where large numbers of Wassermann tests have to be carried out, and there is need for economy in labour and materials, the test may be abridged as follows: tube 4 of the "test" series is omitted and 6 M.H.D. of complement are substituted for 8 M.H.D. in tube 3; tube 2 of the "serum control" may also be omitted, though this may sometimes introduce a difficulty if a serum is anti-complementary; tube 4 of the complement-dose control can be omitted unless the complement is unusually weak.

The mixtures are incubated at 37° C. for one and a half hours, and then to each tube 0·5 c.c. of the haemolytic system is added. The tubes are again incubated for one hour, when the results are noted. Absence of haemolysis in the test series signifies that

EXAMPLES OF THE RESULTS OF WASSERMANN TESTS CARRIED OUT BY THE METHOD DESCRIBED

	Test proper				*Serum Control*		*Antigen Control*	
Tube	1	2	3	4	1	2	1	2
Amount of complement in M.H.D.	2	4	8	12	2	4	2	4
Amount of *undiluted* complement in c.c.	0·012	0·024	0·048	0·072	0·012	0·024	0·012	0·024
Negative serum	Complete lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis
Positive serum	No lysis	No lysis	Partial lysis	Complete lysis	Complete lysis	Complete lysis	–	–
"Weak positive" serum	No lysis	Partial lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis	–	–
"Marked positive" serum	No lysis	No lysis	No lysis	No lysis	Complete lysis	Complete lysis	–	–

Complement dose :—

Undiluted complement in c.c.	0·004 Marked lysis	0·006 Just complete lysis	0·008 Complete lysis

In this experiment the M.H.D. of undiluted complement is 0·006 c.c. both in the initial titration and in the complement-dose control.

fixation of complement has occurred, so that not even 1 M.H.D. is left free. The serum and antigen controls determine any possible anti-complementary action of either of these reagents, and the complement dose control indicates any possible deterioration in the haemolytic value of the complement by dilution and incubation. By carrying out the test in this quantitative way the number of doses of complement fixed by antigen *plus* serum can be estimated—*i.e.* the strength of the reaction. *Known negative and positive sera must always be included in each set of Wassermann tests as controls.*

It must be remembered that specimens of complement from different guinea-pigs vary in fixability and it is advisable, therefore, to use the pooled serum of several animals. With a weakly fixable complement it may even be impossible to elicit positive reactions, while with a strongly fixable complement non-specific effects are rendered more marked. The controls with known negative and positive sera are indispensable. The positive control serum should be a weakly reacting one.

Any anti-complementary effect of the serum or antigen must be allowed for in estimating the fixation by serum *plus* antigen, and the results with known positive and negative sera are to be regarded as standards for comparison with the results given by the sera tested.

The following criteria of positive and negative reactions may be followed: a serum which permits of complete lysis with the same minimum amount of complement as the negative control serum is, of course, negative; a serum which requires at least twice as much complement as the negative control serum to yield complete lysis is to be regarded as positive. Sera which show intermediate effects may be classified as "doubtful." If the serum in question exhibits any degree of anti-complementary effect, allowance must be made for this.

The practical details and interpretation of the results can be learned only by actual acquaintance with the test.

THE WASSERMANN REACTION WITH CEREBRO-SPINAL FLUID

In testing spinal fluid the reaction is carried out as in the case of serum, but with the following modifications :—

(1) The antigen is prepared by emulsifying 1 part of the cholesterolised sheep heart extract with 12 parts of spinal fluid ; 0·5 c.c. of this mixture replaces antigen *plus* serum in the usual test.

The alternative antigen (2) is prepared by mixing 1 part of the cholesterolised extract with 29 parts of the fluid.

(2) In place of saline *plus* serum in the usual serum controls, 0·5 c.c. of spinal fluid is tested.

(3) The cerebro-spinal fluid is not usually heated to 55° C. before the test as in the case of serum.

METHOD II. (*standard volume method*)

HARRISON'S TECHNIQUE MODIFIED BY WYLER[1]

It is not possible to include all the minutiae of detail described by Wyler, and workers who use this method should consult his monograph. The main essentials of reagents and technique, however, are given below.

In this test a standard volume of each reagent is used, and 0·11 c.c. is the amount selected. An ordinary capillary pipette is graduated with mercury[2] as follows : 0·11 c.c. of mercury is accurately measured into a watch glass ; the mercury is then gently sucked into the capillary pipette (held almost horizontally) and a mark made at the upper level with glass-writing ink. The pipette is used with the rubber tube and mouthpiece described on p. 253, or with a rubber teat. Instead of adding different amounts of the same dilution of complement, as in the method previously described, the same amount of different dilutions is taken. For example, suppose the M.H.D. were the standard volume of a 1 in 60 dilution of complement, then 3 M.H.D. would be present in the standard volume of a 1 in 20 dilution, while for 5 M.H.D. a 1 in 12 dilution would be used.

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[1] For full details of this method see "The Wassermann Test," by Wyler, E. J., *Medical Research Council's Special Report Series*, No. 129, 1929 ; *Ministry of Health Reports on Public Health*, No. 67, 1932 ; *J. Path. Bact.*, 1934, **39**, 521.

[2] Measurements could also be made with the graduated pipettes referred to on p. 250.

Antigen—

An alcoholic extract of *human heart-muscle* is prepared similarly to that described on p. 266 (2). For use, place in a wide tube 0·3 c.c. antigen and 0·2 c.c. 1 per cent. solution of cholesterol in absolute alcohol. Add very rapidly 7 c.c. of saline. This 1 in 15 dilution is the antigen suspension referred to in the description of the test proper.

Patient's serum—

This is inactivated and a 1 in 5 dilution in saline is used.

Complement—

Guinea-pig serum (p. 266).

Haemolytic system—

Sheep red corpuscles, 3 per cent., are sensitised with 6 M.H.D. immune body. The cell suspension is standardised by means of a Haldane haemoglobinometer. Either Burroughs Wellcome haemolytic serum, or rabbit *v.* sheep antiserum, can be used.

Titration of complement—

Prepare 2 c.c. of a 1 in 10 dilution of the guinea-pig serum in saline. Set out eight tubes. Add to each respectively, starting from the left, 1 volume of each of the following dilutions of complement prepared from the 1 in 10 dilution : 1 in 30, 1 in 40, 1 in 50, 1 in 60, 1 in 70, 1 in 80, 1 in 90, 1 in 100. To each tube add 2 volumes of saline and 1 volume of sensitised red cells. Prepare a similar series of tubes with the complement dilutions, but instead of adding 2 volumes of saline add 1 volume of antigen suspension and 1 volume of saline. Then add 1 volume of sensitised red cells and shake thoroughly. Incubate both sets of tubes in the water-bath for half-an-hour at 37° C. and read the result. In the first set of tubes (*i.e.* without antigen) the highest dilution showing complete haemolysis is the minimum haemolytic dose. Dilutions of complement equivalent to 2, 3 and 5 M.H.D. are now prepared from the undiluted guinea-pig serum. The second set of tubes (with antigen) indicates whether the antigen suspension has any anti-complementary action.

Test proper—

Four tubes are required for each test, two for serum controls and two for the antigen-serum mixture. The contents of each tube are as follows :—

Tube 1 (serum control) .	1 volume saline. 1 volume patient's serum diluted 1 in 5 in saline. 1 volume complement diluted to 3 M.H.D.
Tube 2 (serum control) .	Same as tube 1, but the complement present is 2 M.H.D.
Tube 3 . . .	1 volume patient's serum diluted 1 in 5 in saline. 1 volume complement 5 M.H.D. 1 volume antigen suspension.
Tube 4 . . .	Same as tube 3, except that complement is 3 M.H.D.

The reagents are added in the following order :—

(1) 1 volume of dilution of patient's serum into all tubes.
(2) 1 volume of saline into tubes 1 and 2.
(3) 1 volume complement dilution 2 M.H.D. into tube 2.
(4) 1 volume complement dilution 3 M.H.D. into tubes 1 and 4.
(5) 1 volume complement dilution 5 M.H.D. into tube 3.
(6) 1 volume antigen suspension into tubes 3 and 4.

The racks are shaken after each set of ingredients is added to the tubes.

The tubes are incubated first at room temperature for thirty minutes and then in the water-bath at 37° C. for thirty minutes, after which 1 volume of sensitised red cells is added, and the tubes are well shaken and replaced in the water-bath at 37° C.. After one or two minutes they are taken out, quickly shaken and replaced. The results are read as soon as the No. 1 tubes and some of the No. 4 tubes, including the No. 4 tube of the negative control, show complete haemolysis—usually in about five minutes.

In addition to the test proper, described above, in which known positive and negative sera are used as controls, an antigen control and a corpuscle control are also carried out at the same time.

Antigen control—

To one tube are added 1 volume saline, 1 volume 2 M.H.D. complement and 1 volume antigen. This is placed in the rack with the test proper, 1 volume of red cells being added later along with the rest of the tubes.

On incubation there should be complete haemolysis within thirty minutes, confirming that the antigen does not absorb more than one dose of complement.

Corpuscle control—

This consists of 1 volume of sensitised red cells with 3 volumes of saline. The red cells are added to the saline at the same time as to the other tubes. The tube is centrifuged when the tests have been read. The supernatant fluid must be colourless, showing that no deterioration of the cells has occurred since the complement was titrated.

METHOD OF RECORDING THE RESULTS OF THE TEST

Symbols—

++ = Complete inhibition of lysis with 3 M.H.D. complement and complete or almost complete inhibition with 5 M.H.D. complement.

+ = Complete or almost complete inhibition of lysis with 3 M.H.D. complement and partial inhibition of lysis with 5 M.H.D. complement.

± = Partial inhibition of lysis with 3 M.H.D. complement ; slight inhibition or complete lysis with 5 M.H.D. complement.

– = Complete lysis with 3 M.H.D. complement.

Interpretation of Symbols—

$\left.\begin{array}{l}++ = \text{Strong positive}\\ + = \text{Positive}\end{array}\right\}$ Diagnostic.

± = Positive in *known* cases of syphilis only. In unknown cases it is regarded as *doubtful*, and as an indication for further investigation.

– = Negative, but the patient is not necessarily non-syphilitic.

Test with cerebro-spinal fluid—

The fluid does not require to be heated.

Into a series of tubes place varying quantities of the cerebro-spinal fluid as follows :—

2 volumes undiluted fluid.
1 volume undiluted fluid.
1 volume 1 in 2·5 dilution.
1 volume 1 in 5 dilution.

To each tube are added 1 volume antigen and 3 M.H.D. complement. As a control another tube with 2 volumes of undiluted fluid and 3 M.H.D. complement, but without antigen, is used. The remainder of the procedure corresponds to the serum test, and the strength of the reaction is judged by the number of tubes, excluding the control, in which a positive reaction occurs. A strongly reacting fluid gives a positive result with the 1 in 5 dilution, while a weakly reacting specimen yields fixation of complement only in the tube containing 2 volumes of undiluted fluid.

Partial or even complete inhibition of lysis in the tube containing 2 volumes of undiluted cerebro-spinal fluid cannot be accepted as positive for diagnostic purposes unless there is also definite inhibition of lysis in the tube containing 1 volume of undiluted fluid.

For the technique of carrying out the test, with dropping pipettes, Wyler's paper (see third reference, footnote, p. 274) should be consulted.

THE FLOCCULATION TEST FOR SYPHILIS

Since the discovery by Michaelis (1907) that precipitation or flocculation occurs when a syphilitic serum is treated with organ extracts, various tests depending on this phenomenon have been described and recommended for the routine serum diagnosis of syphilis, as substitutes for the Wassermann reaction —*e.g.* the Sachs-Georgi reaction, the Sigma reaction of Dreyer and Ward,[1] the Kahn reaction, the Meinicke test, etc.. Most of these have been reviewed in the League of Nations report on the sero-diagnosis of syphilis.[2] The differences in technique depend mainly on the manner in which the organ extract is prepared, and the method by which the resulting flocculation, precipitation, or clarification (Meinicke test) is observed.

The method of the Bacteriology Department, Edinburgh University, may be recommended as a simple and reliable technique. The results are practically parallel to those of the complement-fixation test, and the flocculation reaction may be substituted for it; the serum, however, should be tested within two days after withdrawal of the blood specimen. In treated cases of syphilis the flocculation reaction may be definitely positive when the Wassermann reaction is very weak or negative; sometimes, however, the reverse result is obtained. It is to be noted that the flocculating property of syphilitic serum may be lost if specimens of blood are kept for several days.

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[1] *Lancet*, 1921, ii., 956.

[2] *League of Nations Publications*, 1928, No. C.H.726; and 1931, No. C.H.968.

The "Antigen" is similar to that used for the Wassermann reaction.

20 grams of sheep heart-muscle are ground with sand in a mortar and extracted for four days at room temperature with 100 c.c. of 96 per cent. alcohol. The extract is filtered and 0·25 gram cholesterol is added and dissolved by keeping the extract in a water-bath at 55° C. for half-an-hour. It is then allowed to stand at room temperature for a day and again filtered.

The optimum dilution of this antigen for the test is usually 1 in 18, but may vary for different preparations, and must therefore be ascertained for each new preparation. Likewise the "sensitiveness" must be compared with a known standard so that, if necessary, the antigen can be corrected or standardised.

For details of the method of standardising this antigen, reference should be made to the *Indian Journal of Medical Research*, 1929–30, vol. 17, p. 477: "The Standardisation of Antigen used in the Syphilis Flocculation Reaction," by K. V. Krishnan.

The reaction is carried out in a similar manner to an agglutination test (*vide* p. 250).

The activity of the serum is at a maximum after heating at 54°–56° C.. For the test the patient's serum is therefore heated at 55° C. for half-an-hour and a series of concentrations is prepared in small test-tubes ($3 \times \frac{1}{2}$ in.) as follows (physiological saline solution being used as the diluent):—

①	②	③	④	⑤	⑥
1 in 2	*1 in 4*	*1 in 8*	*1 in 16*	*1 in 32*	*1 in 64*

0·4 c.c. of each concentration is a convenient quantity for the test, and to each tube one-half of this volume (0·2 c.c.) of the antigen suspension is added. If the optimum dilution of the antigen is 1 in 18, then a 1 in 6 suspension is used; it is prepared by mixing rapidly equal quantities of the undiluted antigen and saline solution, allowing the mixture to stand for ten minutes, and then adding sufficient saline to give the required dilution (*i.e.* 1 in 6). The mixtures of serum and antigen are transferred to narrow agglutination tubes, incubated at 37° C. for four hours, and allowed to stand overnight at room temperature, when readings are made of the results. A final reading should be made after thirty-six hours. In reading the results, the tubes should be held in front of a shaded lamp so that they are brightly illuminated without the glare of the lamp reaching the eyes. A control tube containing antigen suspension and saline but no serum should be included. With strongly reacting sera, flocculation may occur even in tube ⑥. With weak sera definite flocculation may occur only in tube ①. "Zone phenomena" are sometimes observed, *e.g.* with sera after three days or more from the time of withdrawal of the blood, flocculation being less with lower dilutions of serum than with higher dilutions. Such zone effects are specially noticeable with sera which give strongly positive Wassermann reactions.

The reaction can be hastened by shaking the mixtures of serum and antigen for five to ten minutes before incubation. This can easily be done after the addition of the antigen to the serum dilutions, by placing the small test-tubes vertically in a shaking machine (such as that used for shaking a Winchester quart bottle). The tubes are held in racks fixed to the carrier of the machine.

The test can, if necessary, be abridged by omitting tubes 5 and 6.

THE KAHN FLOCCULATION TEST

Apparatus required—

(1) Small test-tubes, $3 \times \frac{1}{2}$ in. as used in the Wassermann test; these tubes should be of perfectly clear glass and thoroughly clean.

(2) Flat-bottom glass cylinders, $1\frac{1}{2} \times \frac{1}{2}$ in. for the preparation of the diluted antigen.

(3) 1 c.c. and 0·1 c.c. graduated pipettes as used in the Wassermann test.

(4) Special pipettes: one graduated from the tip to deliver 0·0125, 0·025 and 0·05 c.c. respectively; the other with one graduation to deliver 0·15 c.c.

(5) Suitable racks for the tubes.

Reagents—

(1) Patient's serum—at least 0·5 c.c. required.

(2) Antigen—" Bacto " Kahn standard antigen which can be obtained commercially[1] is recommended as being very satisfactory in stability and sensitivity. Alternatively it can be made from " Bacto " Beef Heart.[1]

(3) 0·85 per cent. sodium chloride in distilled water.

(4) Control sera—at least four should be included in any set of tests ; these should be selected according to previous results as follows : (*a*) Negative, (*b*) $+^1$, (*c*) $+^2$, (*d*) $+^3$ (*vide infra*). All sera tested are heated at 55° C. for 30 minutes before testing.

Dilution of antigen—

The antigen is diluted with saline in the proportions prescribed for the preparation : usually 1 : 1·1.

(1) Ascertain the total volume of diluted antigen required for the set of tests by multiplying the number of sera by 0·0875 c.c. (the volume of diluted antigen required for one serum) and adding to this figure 0·3 c.c. for loss in pipetting, etc.. Not more than is sufficient for 40 tests should be made up at one time.

(2) Pipette *separately* into each of two small cylinders (referred to above) the volumes of normal saline and undiluted antigen required to yield in the prescribed proportions the total bulk of diluted antigen.

(3) Add the saline from one cylinder rapidly to the antigen in the other and mix by pouring from one cylinder to the other five or six times.

The diluted antigen should be used for the test not less than 10 minutes and not more than 30 minutes after mixture.

The *test* for each serum is set up as follows :—

Tube . .	1	2	3
Add *diluted antigen* .	0·05 c.c.	0·025 c.c.	0·0125 c.c.
„ *serum* . .	0·15 c.c.	0·15 c.c.	0·15 c.c.

The tubes are shaken by hand or preferably in a shaking machine for 3 minutes (*vide* p. 280). (After shaking incubation in a water-bath at 37° C. for 15 minutes or an incubator at 37° C. for 20 minutes is advantageous.)

Then add *saline* .	1·0 c.c.	0·5 c.c.	0·5 c.c.

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[1] From Difco Labs. Inc., Detroit, Michigan, per Baird & Tatlock (London) Ltd. For full details of the test and the preparation of the antigen see *The Kahn Test* by R. L. Kahn, 1928, Baltimore.

Readings are now made.

The following *antigen control* is included in each set of tests :

Tube	1	2	3
Add *diluted antigen*	0·05 c.c.	0·025 c.c.	0·0125 c.c.
,, *saline*	0·15 c.c.	0·15 c.c.	0·15 c.c.
Incubate as above.			
Shake tubes as above.			
Then add *saline*	1·0 c.c.	0·5 c.c.	0·5 c.c.

Reading of results.—The tubes should be held in a sloped position and the fluid viewed (if necessary with an 8 × hand lens) in a strong light against a dark background.

The following results may be observed in individual tubes :

$0 =$ the fluid remaining uniformly opalescent.

$+^1 =$ minute floccules just visible to the naked eye throughout the fluid.

$+^4 =$ large floccules sedimenting completely in the tube.

$+^2$ and $+^3 =$ intermediate degrees of flocculation.

The interpretation of results is illustrated as follows :—

Tube		1	2	3	Result stated as	Diagnostic interpretation
Serum	A	0	0	0	0	Negative
,,	B	$+^4$	$+^3$	$+^3$	$(+^{\frac{10}{3}})+^3$	Strong positive
,,	C	$+^3$	$+^2$	$+^2$	$(+^{\frac{7}{3}})+^2$	Positive
,,	D	$+^2$	$+^1$	$+^1$	$(+^{\frac{4}{3}})+^1$	Weak positive
,,	E	$+^1$	0	0	$(+^{\frac{1}{3}})+^{<1}$	Doubtful negative

See **Appendix.**—Kahn Verification Test.

THE COMPLEMENT-FIXATION TEST IN TUBERCULOSIS

This reaction has been utilised in the diagnosis of certain cases of tuberculosis, and also as an index of the activity of the disease.

The principle of the test is that governing complement-fixation reactions generally, and a method corresponding to that used in the Wassermann reaction is employed. The *antigen* consists of tubercle bacilli and their products. Various antigen preparations have been advocated, such as that of Besredka, prepared by growing tubercle bacilli in egg-broth,[1] or the Boquet-Nègre antigen which is prepared as follows : (1) 6 weeks' old glycerol broth cultures of human and bovine tubercle bacilli are killed by autoclaving at 120° C. for 30 minutes ; (2) the cultures are filtered through paper and washed with distilled water on the filter paper ; and then (3) dried in an incubator or desiccator ; (4) the dried bacilli are treated with acetone (1 c.c. for each 0·01 gram of dried bacilli) for 24 hours, and then dried again ; (5) the bacilli are now treated with pure methyl alcohol (1 c.c. for each 0·01 gram bacilli) and extracted for 10–12 days at 37° C., the container being repeatedly shaken ; (6) after centrifuging, the supernatant fluid constitutes the antigen, which is used in a dilution of 1 in 20 in saline. The antigen should be kept in the dark. Precipitates which may form in the cold are re-dissolved by short heating at 45°–50° C..

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[1] *Compt. Rend. Acad. d. Sci.*, 1913, **166**, 1633.

Antigens prepared from the tubercle bacillus may yield positive reactions with strongly-positive Wassermann sera ; a Wassermann test, therefore, should also be carried out.

THE COMPLEMENT-FIXATION TEST IN GONORRHOEA

The general technique of the test is very similar to that of the Wassermann test described on p. 274.

Antigen.—Various types of antigen prepared from cultures of the gonococcus have been used. The antigen recommended is that devised by Price,[1] who claims that in the first week of the disease 27 per cent. of positive results are obtained, rising to 46, 70, 89 and 100 per cent. after two, three, four and five weeks respectively.

This antigen is made as follows : the gonococcus is grown on hydrocele agar in a Roux flask at 37° C. for twenty-four to forty-eight hours. The culture is then washed off into a cylinder with 100 c.c. saline solution. 1 c.c. of N/1 NaOH is added, and the cylinder placed in a 37° C. water-bath for two hours, when most of the organisms will have become dissolved. The fluid is filtered through sterile lint to remove any gross insoluble matter. To the clear filtrate is added 1·5 c.c. N/1 HCl and the cylinder is replaced in the water-bath. White flocculi appear after ten to twenty minutes, and these are centrifuged at high speed and the deposit suspended in 4 c.c. normal saline. N/10 NaOH is added to the suspension drop by drop until pH 7·5 is reached. After vigorous shaking the suspension undergoes solution. 1 c.c. of 1 per cent. formol-saline is now added and the fluid is filtered through lint. The solution is then ampouled in 5 c.c. amounts and heated at 56° C. for two hours. This colloid fluid constitutes the antigen, and is used in the test in a dilution of 1 in 30. Each batch of antigen, however, must be titrated before being put into use.

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[1] I. N. O. Price, Monograph published by the London County Council, No. 2995, 1933 (obtainable from King & Son, London); see also *J. Path. Bact.*, 1932, **35**, 635.

Haemolytic system.—3 per cent. sheep cells *plus* 5 M.H.D. haemolytic immune body.

Complement.—Prepared and titrated as for the Wassermann test (p. 275). Along with this titration, antigen and serum control tests are also made.

Test Proper.—This is similar in technique to Wyler's method for the Wassermann test, a constant-volume method being used. One volume of serum and one volume of diluted antigen are tested with 3 and 5 M.H.D. of complement respectively, together with a serum control containing 3 M.H.D. complement. The mixtures are well shaken, and placed in a 37° C. water-bath for one hour. Thereafter, one volume of the haemolytic system is added, the tubes are well shaken and again placed in the water-bath. Results are read when the normal serum controls are completely haemolysed.

No haemolysis with 5 M.H.D. complement is denominated a + + result; haemolysis with 5 M.H.D., but no haemolysis with 3 M.H.D. +; complete haemolysis with 3 M.H.D., negative.

For full details Price's Monograph should be consulted.

PREPARATION OF BACTERIAL VACCINES

The method to be described refers mainly to the preparation of vaccines on a small scale, such as *autogenous vaccines*—*i.e.* consisting of the organism or organisms isolated from a particular patient, and used for the treatment of the case (*vide* p. 32).

Prophylactic vaccines. — In preparing stock bacterial vaccines for prophylactic use, *e.g.* typhoid - paratyphoid (T.A.B.) vaccine, it is essential that the strains used should be carefully selected, as pathogenic bacteria when maintained in laboratory culture for any length of time may undergo variation in antigenic characters and so lose their specific immunising properties (*vide* p. 42). Further references to this question are made later in Part III. The manufacture of vaccines on a large scale is beyond the scope of this book.

The organism must be isolated in pure culture, and then several cultures are made on appropriate solid medium so as to yield sufficient growth after twenty-four to forty-eight hours' incubation, according to the amount of vaccine to be prepared and the abundance of the growth on the particular medium. The growth is emulsified in sterile saline solution (0·85 per cent. sodium chloride) so as to form a fairly dense suspension. This should be free from fragments of medium ; if present they can be removed by centrifuging the suspension for two or three minutes, or by allowing them to sediment by gravity, and then decanting the supernatant fluid. The bacterial suspension must be rendered as uniform as possible by shaking in a tube or bottle with glass beads. A special shaking machine is generally used for this purpose. *All manipulations involved in preparing the suspension must be carried out with strict precautions to avoid contamination.*

STANDARDISATION

It is necessary at this stage to estimate the *approximate* number of bacteria per c.c. of the suspension. Various methods are available for this purpose. Those recommended are :

1. *Haemocytometer Method.*—Use a Thoma cell or the central portion of any of the rulings on the Bürker pattern of haemocytometer ; make a 1 in 20 dilution of the suspension—0·1 c.c. suspension + 1·8 c.c. saline + 0·1 c.c. centrifuged Löffler's methylene blue, adding two loopfuls of undiluted formalin to kill the organisms ; mount a drop on the haemocytometer stage as in blood-counting and enumerate the organisms in 20–50 small squares in different parts of the cell ; determine the average number per square and multiply this by 80,000,000 ; the result is the approximate number of bacteria per c.c. in the original suspension.

2. *Comparison with Standard Opacity Tubes* (Brown).[1] —This consists in comparing the opacity of the suspension with that of a series of ten standard

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[1] See *Indian J. Med. Research*, 1919, 7, 238-250.

tubes containing different dilutions of suspended barium sulphate. In making comparisons the bacterial suspension should of course be placed in a tube similar to the standards. The matching is facilitated by reading printed letters through the suspensions.

The Table gives the numerical equivalents of the opacity standards for certain organisms according to Cunningham and Timothy.[1]

SHOWING THE RELATION OF OPACITY TO THE NUMERICAL EQUIVALENT OF VARIOUS BACTERIA ESTIMATED BY MEANS OF THE HAEMACYTOMETER METHOD

Opacity Tube No.	*Staphylococcus aureus*	*Streptococcus, haemolytic*	*Pneumococcus*	*Gonococcus*	*B. coli*	*B. typhosus*	*B. paratyphosus B*	*D. catarrhalis (on ord. agar)*	*B. influenzae (Pfeiffer)*
10	3789	3043	7053	3578	3787	4577	4171	3611	11396
9	3410	2739	6348	3220	3408	4119	3754	3250	10256
8	3031	2434	5642	2862	3030	3662	3337	2889	9117
7	2652	2130	4937	2505	2651	3204	2920	2528	7977
6	2273	1826	4232	2147	2272	2746	2503	2167	6838
5	1895	1522	3527	1789	1894	2289	2086	1806	5698
4	1516	1217	2821	1431	1515	1831	1668	1444	4558
3	1137	913	2116	1073	1136	1373	1251	1083	3419
2	758	609	1411	716	757	915	834	722	2279
1	379	304	705	358	379	458	417	361	1140

The figures represent millions per c.c.

Standard opacity tubes with the corresponding tables are supplied by Burroughs Wellcome & Co.

STERILISATION OF THE BACTERIAL SUSPENSION

The suspension is sterilised at relatively low temperatures—*e.g.* 60° C. for one hour in a water-bath. To ascertain whether the organisms have been killed, several loopfuls are transferred to a tube of *suitable* medium and incubated for forty-eight hours. (For further sterility tests, *vide infra.*)

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[1] See *Indian J. Med. Research*, 1924, **11**, 1253.

PREPARATION OF THE VACCINE FOR ADMINISTRATION

Any series of graduated doses consisting of a certain number of organisms (usually computed in millions) can be prepared in volumes of 1 c.c. by making appropriate dilutions in carbol-saline (0·85 per cent. sodium chloride+0·5 per cent. phenol) from the original standardised suspension. Graduated pipettes, as used in serological work, are employed for the purpose. The dilutions are made in sterile tubes and each dose is transferred to a sterile vaccine ampoule which is then sealed.

The most convenient method of supplying the vaccine for actual use is to prepare from the stock suspension, concentrations of 50, 100, 500 or 1000 million organisms per c.c. (according to the doses required) in quantities of 20 c.c. The dilutions are placed in 25 c.c. " vaccine bottles "[1] with special tightly-fitting thick rubber caps which are covered with a layer of paraffin wax, or in 1 oz. bottles with perforated screw-caps like that of the blood-culture bottle (*vide* p. 184), and covered with a viskap before issue. The required dose can be obtained by puncturing the cap with the hypodermic syringe and withdrawing the appropriate amount.

When a vaccine representing more than one type of organism is required—*e.g.* from mixed infections—pure cultures of each organism must be obtained and separate standardised suspensions prepared. Appropriate concentrations of each are then combined in the final preparation.

In preparing dilutions from the stock vaccine all manipulations, etc., must be carried out with strict precautions to prevent contamination. Pipettes, tubes, ampoules, bottles, caps, etc., must be absolutely sterile.

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[1] A very suitable container is the " Clinbritic " vaccine bottle which is fitted with a skirted type of rubber cap and a screw-on superimposed bakelite cap. This bottle is obtainable from Britton, Malcolm & Waymark Ltd., 38 Southwark Bridge Road, London, S.E.1.

Before supplying the diluted vaccine it is essential to carry out further sterility tests with the contents of one of the ampoules, or 2 c.c. withdrawn from the bottled vaccine with a syringe. One-half of this sample is tested for aerobic organisms and one-half for anaerobes by appropriate cultural methods.

The Regulations under the Therapeutic Substances Act applicable to the " manufacture for sale " of various therapeutic substances, including vaccines, lay down *standard sterility tests* which should be followed :

" *The tests shall be made on fluid media, the quantity of medium contained in each tube or other vessel used in the test being such as to secure that any phenolic antiseptic present in the sample is diluted to less than* 0·01 *per cent.*

In the case of a test for aerobic organisms the medium shall consist either of a meat extract with the addition of 1 *per cent. of peptone, or of such an equivalent as can be prepared by the tryptic digestion of muscle. After the final sterilisation the hydrogen-ion concentration of the medium shall be between the limits represented by* $pH=7{\cdot}2$ *and* $pH=7{\cdot}8$.

In the case of a test for anaerobic organisms the medium shall consist of a nutrient broth similar to that used in testing for aerobic organisms, with the addition of heat coagulated muscle of an amount sufficient to occupy a depth of not less than 1 *centimetre at the bottom of the tube. After the final sterilisation the hydrogen-ion concentration of the medium shall be between the limits represented by* $pH=7{\cdot}2$ *and* $pH=7{\cdot}8$. *Before the test inoculation the medium shall be heated to* 100° C. *for a period sufficient to free it completely from dissolved oxygen, and then cooled to* 37° C. *or lower.*

The inoculated tubes shall be incubated at 37° C. *for five days. . . .*"

If a vaccine has been prepared from an organism which does not grow readily in ordinary media, a similar test must be carried out with media specially suitable for the growth of the particular organism, or the vaccine may be tested by injection of an animal of a species known to be susceptible to infection by that organism.

LABORATORY CENTRIFUGES

The use of the centrifuge is to separate cells, organisms, or other particles from a fluid suspension. On standing, the cells, etc., will slowly sediment by gravity, but this sedimentation can be greatly accelerated by means of the centrifuge. The "relative centrifugal force," which indicates the degree to which the normal force of gravity is increased, depends, amongst other factors, on the radial distance of the centrifuged material from the central axis, and on the square of the number of revolutions per minute. It should be appreciated also that the shape and size of the particles, the viscosity and surface tension of the fluid, and the difference in specific gravity between the particles and fluid, play an important part in the process. For example, when defibrinated blood is being washed (*vide* p. 268), the first sedimentation of the corpuscles from the viscous serum of high specific gravity takes much longer than when the corpuscles are suspended in saline.

Types of Centrifuges.—For general laboratory work they are of three types: (1) Hand-driven; (2) Water-driven; (3) Electric.

Hand-driven centrifuges have two or four 15 c.c. conical metal "buckets" carrying the glass centrifuge tubes (*vide infra*), and are chiefly used by medical practitioners or in small laboratories where electric power or water is not available. The speed attained rarely exceeds 2000 r.p.m. while the motion is jerky and the column supporting the buckets tends to vibrate. Moreover, the buckets come to rest quickly when the drive ceases, thereby tending to disturb the sediment. The hand centrifuge is sometimes convenient when separating serum in a Wright's capsule (*vide* p. 249).

Water-driven centrifuges utilise a water turbine to the spindle of which the head is connected. At least 30 lbs. per sq. in. pressure is required to work the machine satisfactorily. The head usually consists of a circular plate with a flange, under which the buckets are drawn when the centrifuge is in action. The water-driven centrifuge is not usually made to accommodate big loads, four 5, 10 or 25 c.c. buckets being the usual equipment. With 30 lbs. water pressure speeds of about 2000 r.p.m. are obtained. The machine is quiet, requires no attention, and is useful where a number of small quantities have to be centrifuged.

Electric centrifuges may be obtained for various quantities of fluid up to 1 litre. The machine consists of a motor, with a vertical spindle to which the head is attached. An assortment of heads may be used according to the amounts and number of the specimens. The portable type of electric centrifuge mounted on rubber cushions on a stand with rubber castors is very useful. Speeds up to 4000 r.p.m. are usually obtained. For greater speeds more powerful and strongly designed machines, which are therefore more costly, are required. With these centrifuges the head is usually mounted on a firm stand and is belt-driven from a separate motor.

Speeds up to 3000 r.p.m. are sufficient for ordinary laboratory use. Electric centrifuges should be fitted with some form of "no-volt release" so that if the current is interrupted (*e.g.* by a blown fuse or from a main switch) and the machine stops, it will not start again when the current is restored. Otherwise under such conditions the motor will start violently, with damage to the machine and its contents.

The *metal buckets* which hold the glass centrifuge tube are of various capacities. The buckets for conical glass tubes should be cylindrical and not conical, as the conical tubes, being hand-made, are not all of the same slope and therefore may not fit accurately into a conical bucket, but touch only at one place so that on centrifuging, the tube is broken and the contents lost.

Centrifuge tubes.—The thickness of the wall of the tube varies according to the capacity, but tubes of the same size should have a uniform thickness of wall so that when balanced they contain approximately the same amount of fluid.

The tubes are usually plugged with cotton-wool and sterilised in the hot-air oven. When used in the centrifuge, however, the cotton-wool plugs may be drawn to the bottom of the tube unless the upper portion of the plug is folded over the mouth of the tube and secured with a rubber band. Even when the cotton-wool plug is secured in this way cotton fibres become detached owing to the speed of the centrifuge and are seen microscopically in the deposit.

The most convenient method is the following :—

Instead of a cotton-wool stopper, a screw-cap without a washer is placed over the mouth of the tube, the size being that of a loose fit. (For the ordinary 15 c.c. conical tubes the M.2 screw-cap of a ¼ oz. "bijou" bottle is convenient.) Each centrifuge tube with cap in position is wrapped individually in kraft paper and sterilised in the hot-air oven. During centrifuging the cap is kept on the tube and the contents remain sterile. Moreover, the mouth of the centrifuge tube is kept sterile and the supernatant fluid can be poured off without risk of contamination.

It is essential that each centrifuge tube and bucket should be balanced accurately by a similar tube and bucket diametrically opposite on the circumference of the head. Various types of balances are catalogued for this purpose, but usually they are made only for one size of tube. Where several heads and different sizes of bucket are used the simplest and most convenient type is the commercial "butter balance" of the Beranger type having two flat pans, one being of porcelain. The larger buckets stand upright on the flat pans, while for the 15 c.c. buckets a simple holder may be made from a wooden container such as is used for transmitting test-tubes by post. The top is cut off, leaving the holder $1\frac{1}{2}$ in. high, which is then nailed to a piece of thin wood (plywood) $\frac{3}{16}$ in. thick and $1\frac{3}{4}$ in. square to serve as a base. Two of these are required and they can be balanced accurately with fine shot or small pieces of sheet lead.

When, for example, a sample of urine has to be centrifuged, a sterile centrifuge tube is filled to within $\frac{1}{2}$ in. of the top, the metal cap is fitted, and the tube is placed in a holder on the balance. A similar centrifuge tube is filled with water and placed in a holder, and the cap is laid on the balance pan beside it ; by means of a rubber teat and capillary pipette water is added or removed from the second tube until both tubes are accurately balanced. The metal cap is placed over the second tube and both caps are inserted opposite each other in the centrifuge head. The lid of the centrifuge is secured and the centrifuge *slowly* started. When the head is rotating freely, the speed is gradually increased, with the rheostat or by increasing the water pressure, according to the motive power, until the desired speed is reached. After the prescribed time the current is switched off or the water power shut off, and the head comes slowly to rest. When all movement has ceased the tubes are lifted from the buckets. Tubes of the larger sizes should be balanced in their metal buckets, which are then fitted into the centrifuge head.

It is essential that the hinged or detachable lid, which is fitted to most centrifuges, should be closed during centrifuging, otherwise a decrease in the speed of revolution due to "windage" will ensue.

The makers' instructions for oiling and overhaul of the machines should regularly be carried out.

The special centrifuges used in connection with virus studies (p. 596) do not come within the scope of this book.

Angle Centrifuge.—In this type of instrument the tubes, instead of being allowed to rotate in a horizontal plane, are fixed at an angle (from 20° to 45°) on the rotating head. The centrifuge is driven by a "universal" motor (suitable for both direct and alternating current and different frequencies). The usual speed is 3000–4000 r.p.m. The advantage of the angular position is that particulate matter is rapidly separated and concentrated, with saving of time and current. Thus, particles have only to traverse a short distance before deposition on the side of the tube. The tubes are encased in a nickel-plated " bowl," which in its rotation offers very slight resistance to air ; this also reduces consumption of power by the motor. The centrifuge has rubber feet and stands on the laboratory bench without being bolted or fixed. It is very convenient for ordinary routine purposes and has been used also for separating virus bodies.

COLD STORAGE

It is essential to have some form of cold storage in the laboratory for the preservation of blood, serum, culture media, vaccines, etc.. The ordinary ice-chest in which the temperature is reduced by a block of ice renewed at frequent intervals has been commonly employed. Its use, however, is not recommended, as it really does not produce a sufficiently low or uniform temperature ; moreover, in hot weather a store of ice sufficient for the week-end cannot be placed in the ice-chamber, while the receptacle for the water from the melted ice is usually inadequate for more than a small block.

It is recommended, therefore, that some form of mechanical refrigeration be used, and refrigerators are available in a large number of sizes from 1½ cubic feet capacity to cold storage rooms of several thousand cubic feet. For the smaller laboratory one of the domestic refrigerators of 4–6 cubic ft. capacity is suitable, while larger laboratories require a correspondingly larger instrument, or an insulated cold room with the refrigerating plant outside. Mechanical refrigerators can be obtained to work with electricity, gas or oil, and most of them have provision for making small quantities of ice. The temperature should be maintained between 4°–5° C. (39°–41° F.). It should never be so low as to cause freezing, as this may be detrimental to vaccines, bacterial suspensions, red cells and certain sera containing a preservative.

It should be noted that with the domestic type of refrigerator an accumulation of ice, due to freezing of water vapour, surrounds the freezing unit and at intervals (about 10–14 days) it is necessary to " de-frost " to remove this ice. The contents of the refrigerator are removed, the current (or gas) turned off and the doors opened. The melted water from the ice is caught in a suitable receptacle. When the ice is melted the interior of the refrigerator is wiped with a cloth, the contents replaced, and the refrigerating unit started again.

CHAPTER IX

BACTERIOLOGICAL EXAMINATION OF WATER AND MILK; TESTING OF ANTISEPTICS; TESTING OF SURGICAL CATGUT FOR STERILITY; COLLECTION AND FORWARDING OF SPECIMENS FOR BACTERIOLOGICAL EXAMINATION

BACTERIOLOGICAL EXAMINATION OF WATER

From the hygienic standpoint, the bacteriological examination of water resolves itself into the determination of the presence or absence of any serious excretal contamination.

Under certain conditions, *B. coli* represents the most reliable indicator of such pollution.

As this group of organisms is derived from the intestine of various animals, even water supplies far removed from human contamination may contain *B. coli* in small numbers. In water grossly polluted with excretal matter (*e.g.* sewage), *B. coli* is present in large numbers. If it is to be accepted as an indicator of impurity, the test for its presence must be carried out on a quantitative basis, so that the *B. coli* content of the water can be estimated.

This group includes a large number of different types. Some of these (" typical " or " faecal ") are more prevalent in the intestine than others ("atypical") and obviously the former are of more significance as indicators of recent faecal contamination. Therefore, in carrying out the test for *B. coli* in water, it is essential to determine whether the strains present are "typical" or "atypical" (*vide* pp. 299, 302).

The presence of streptococci and sporing anaerobic bacilli—e.g. *B. welchii*—is of additional significance in determining water purity. The occurrence of streptococci is strong evidence of faecal pollution, but their absence does not exclude such impurity. The intestinal sporing anaerobes, being highly resistant, do not by themselves indicate a recent or serious pollution.

The enumeration of the total viable bacteria in a water specimen is also a useful supplementary test in determining its purity, but this test is of little value by itself.

The demonstration of pathogenic bacteria (e.g. *B. typhosus*) would obviously constitute the most direct proof of a dangerous impurity, but pathogens, if present, are usually so scanty that the technical difficulty of their isolation makes this test impracticable for ordinary purposes.

The routine tests generally used in the bacteriological examination of water are:

(1) Enumeration of the viable bacteria.

(2) The quantitative *B. coli* test.

Collection of specimens.—Specimens are taken in bottles, of approximately 230 c.c. capacity, with ground-glass stoppers having an overhanging rim to protect the mouth of the bottle from extraneous contamination; they are sterilised by dry heat, the stopper and neck of the bottle being covered by two layers of kraft paper (*vide* p. 90). Alternatively, 6 oz. screw-capped bottles can be used; the capped bottle is wrapped in kraft paper and sterilised in the autoclave. The opening and closing of the bottle and its handling in the process of collecting a sample must be carried out with meticulous care to avoid any bacterial contamination from an outside source, including the hands of the person taking the sample. When water is drawn from a tap, the mouth of the tap should be flamed, *e.g.* with a blow-lamp or spirit lamp, and the water allowed to run for five minutes before filling the bottle. In the case of streams, rivers and lakes, the bottle is filled by dipping the mouth (with the stopper in position) below the surface, and then removing the stopper under water with forceps. This avoids the collection of surface water, which may contain a good deal of decomposing vegetable matter. When a sample is to be obtained from a depth, a bottle weighted with lead is used, having two cords attached —one to the neck, the other to the stopper; the bottle is lowered to the required depth, and is filled by jerking out the stopper by means of the attached cord; the bottle is then quickly raised to the surface and re-stoppered.

When a certain length of time (three hours or more) must elapse before the laboratory examination can be carried out, the bottles should be kept on ice. Special insulated boxes for the purpose can be obtained, and are essential where specimens have to be transported some distance.

Immediately before testing, the water sample should be mixed by inverting the bottle several times.

Enumeration of viable bacteria.—With a sterile graduated pipette place 1 c.c. water in a sterile Petri dish (4 in. diameter), and add 10 c.c. nutrient agar (standardised to pH 7·2), melted and cooled to 45°–50° C.; mix thoroughly and allow to solidify. The agar should be as transparent as possible.

If the water is suspected of contamination, add a smaller quantity—*e.g.* 0·1 c.c.—and in dealing with specimens of uncertain purity it is advisable to make a series of plate cultures with varying quantities of the water. Thus serial dilutions may be made from the sample—*e.g.* 1 in 10, 1 in 100—as in examination of milk (*q.v.*) and 1 c.c. quantities of these plated.

Make *duplicate* plates from each volume tested and incubate one at 37° C. for two days, the other at 20–22° C., for three days. Nutrient gelatin in place of agar has sometimes been used for determining the number of organisms viable at 20°–22° C., but it is preferable to use agar for both counts.

Count the colonies that develop in the medium. To aid counting, divide the plate into sections by ruling on the glass with a grease pencil. Count the colonies in each section (using a hand lens if necessary to detect small colonies) and add the numbers. Each colony may be taken to represent one viable bacterium in the original specimen.

The number of viable bacteria in the average municipal water supply—*i.e.* after filtration—should not usually exceed 100 per c.c. A count of 1000 per c.c. or more may arouse suspicion regarding the purity of a water supply, but does not necessarily signify dangerous pollution ; thus, an increase of organic matter in the summer season may lead to bacterial multiplication and an unusually high bacterial count. On the whole the results of such enumeration are of relatively little value by themselves in estimating the hygienic purity of a water though, when regular observations of this nature are made on the same water supply, a high count on a particular occasion may draw attention to some condition of the supply requiring further investigation.

In uncontaminated water, since the organisms present are mainly saprophytes, the number of colonies developing at 20°–22° C. is usually much greater than at 37° C., the ratio being higher than 10 : 1. A contaminated water may yield a ratio less than this —*e.g.* 10 : 3.

B. Coli Test—

The object of this test is to ascertain the number of *B. coli* in a given volume of water or the smallest amount of water which contains this organism. The estimation is usually made by adding varying quantities of the water (from 0·1 c.c. to 50 c.c.) to bile-salt-lactose-peptone water (with an indicator of acidity) contained in bottles with inverted tubes to show the formation of gas, acid and gas formation (a " positive " result) indicating the growth of *B. coli* (*vide* pp. 121, 299). In this way it may be possible to state the smallest quantity of water containing *B. coli* and thus to express the degree of contamination with this organism. Further, by isolating the coliform bacillus so demonstrated and determining its characters, the smallest amount of water containing *typical B. coli* can also be stated.

This method, however, involves "random sampling" and it is not sufficient to test one sample only of each quantity in the series, for in this way misleading results are obtained. Thus, if two samples of a given quantity were tested one might be positive, the other negative, and a certain quantity might give a positive result while a larger volume is negative. The difficulty can be got over to some extent by duplicating the samples of each quantity in the series and only reporting that a certain volume contains *B. coli* if both samples show it and if all the larger volumes tested yield a positive result. Greater accuracy can only be obtained by increasing the number of samples of each quantity so that an average result can be stated. It has been shown that if one 50 c.c., five 10 c.c. and five 1 c.c. volumes, or five 10 c.c., five 1 c.c. and five 0·1 c.c. volumes are tested, the probable number of coliform bacilli in 100 c.c. can be computed according to the various combinations of positive and negative results, tables compiled by McCrady being used for the purpose. This is the method recommended for routine use.

Media required :

(1) 1 per cent. peptone water containing 0·5 per cent. sodium taurocholate, 1 per cent.

lactose, and neutral red or Andrade's solution as indicator (*vide* p. 121).

(2) Same constituents as (1), but in double concentration.

Measured amounts (*vide infra*) of these media are put up in sterile bottles stoppered with cotton-wool plugs or with screw-caps, and containing an inverted tube for indicating gas production. The size of the bottle varies with the quantity of medium and water to be added to it.

The medium, after bottling, is sterilised at 100° C. for twenty minutes in the steam steriliser on three successive days.

With sterile graduated pipettes the following amounts of water are added :—

One 50 c.c. quantity to 50 c.c. double strength medium
Five 10 c.c. quantities each to 10 c.c. double strength medium
Five 1 c.c. ,, ,, 5 c.c. single ,, ,,
Five 0·1 c.c. ,, ,, 5 c.c. ,, ,, ,,

This range of quantities may be altered according to the likely condition of the water examined ; thus, the 50 c.c. quantity is included when testing filtered or chlorinated water, and in this case it is unnecessary to examine 0·1 c.c. volumes.

The bottles are incubated for forty-eight hours and those showing acid and gas formation (" positive reaction ") are noted. This indicates the growth of coliform bacilli, though further testing is required to determine whether these are " typical." The examination up to the present stage is often designated the " presumptive " *B. coli* test. McCrady's tables (one of which is quoted on p. 300) are now referred to, and according to the various combinations of positive and negative results, the probable number of *B. coli* in 100 c.c. of the water can be stated (see " The Bacteriological Examination of Water Supplies," Report No. 71, Ministry of Health, 1939).

To ascertain whether the coliform bacilli detected in the presumptive test are typical, sub-inoculations are made on MacConkey plates from those bottles which show acid and gas production. The plates are incubated for twenty-four hours, red colonies are picked off on to agar slopes, and the resulting pure growths are put through the following tests :—

Fermentation of *Inositol*
Production of *Indole*
Voges-Proskauer reaction
Methyl-red reaction
Citrate utilisation
Liquefaction of *Gelatin*
(*vide* pp. 425, 427)

The fermentation of lactose is usually assumed from the presumptive test, but should be confirmed.

The common types of *B. coli* of direct excretal origin show the following reactions :—

Lactose	+	(acid and gas)
Inositol	—	
Indole	+	
Voges-Proskauer reaction	—	
Methyl-red reaction	+	
Citrate utilisation	—	
Gelatin	—	

A method has been advocated for counting "faecal" *B. coli* in water without the necessity for plating and examining pure cultures as described above. This depends on the ability of "faecal" coliform bacilli to produce gas when growing in a bile-salt-lactose-peptone-water at 44° C., the atypical coliform bacilli lacking this property (*vide* p. 426, Eijkman reaction). Either of the following procedures may be adopted. (1) The various quantities of water are added to the bile-salt-lactose medium, as described above, *in duplicate*, one set of bottles being incubated at 37° C., the other at 44° C. for two days. The former will yield a count for coliform bacilli irrespective of their type ; from the latter a count can be made similarly for "faecal" *B. coli*. (2) After the usual presumptive *B. coli* test subcultures are made from all the bottles showing acid and gas, into tubes or bottles of the same medium (single strength) as used for the original test, and these are incubated at 44° C. for two days. Those yielding gas may be regarded as containing " faecal " *B. coli*, and a computation of the number in 100 c.c. of water can be made as before.

It has been stressed that incubation at 44° C. must be carried out by keeping the bottles or tubes in a thermostatically controlled water-bath which does not deviate more than 0·5° C. from 44° C.. An incubator regulated at 44° C. is not considered satisfactory for maintaining the cultures at this temperature.

PROBABILITY TABLE (ACCORDING TO McCRADY)

QUANTITY OF WATER	50 c.c.	10 c.c.	1 c.c.		
No. of samples of each quantity tested	1	5	5		
Number giving positive reaction (acid and gas).	0	0	0	0	Probable number of *B. coli* in 100 c.c. of water.
	0	0	1	1	
	0	0	2	2	
	0	1	0	1	
	0	1	1	2	
	0	1	2	3	
	0	2	0	2	
	0	2	1	3	
	0	2	2	4	
	0	3	0	3	
	0	3	1	5	
	0	4	0	5	
	1	0	0	1	
	1	0	1	3	
	1	0	2	4	
	1	0	3	6	
	1	1	0	3	
	1	1	1	5	
	1	1	2	7	
	1	1	3	9	
	1	2	0	5	
	1	2	1	7	

PROBABILITY TABLE—*continued.*

QUANTITY OF WATER	50 c.c.	10 c.c.	1 c.c.		
No. of samples of each quantity tested	1	5	5		
Number giving positive reaction (acid and gas).	1	2	2	10	Probable number of *B. coli* in 100 c.c. of water.
	1	2	3	12	
	1	3	0	8	
	1	3	1	11	
	1	3	2	14	
	1	3	3	18	
	1	3	4	20	
	1	4	0	13	
	1	4	1	17	
	1	4	2	20	
	1	4	3	30	
	1	4	4	35	
	1	4	5	40	
	1	5	0	25	
	1	5	1	35	
	1	5	2	50	
	1	5	3	90	
	1	5	4	160	
	1	5	5	180 +	

According to the older system of notation, these results can be stated alternatively as "*B. coli* present in . . . c.c.," the number of c.c. being 100 divided by the figure in the last column ; thus, if the number of *B. coli* in 100 c.c. is 5, the result can be stated as "*B. coli* present in 20 c.c."

It must be recognised that it is hardly possible to set up fixed bacteriological standards for water purity from the hygienic standpoint, and it has been emphasised in the Ministry of Health's report (cited p. 298) that the aim should rather be to establish a standard for each water supply on the basis of frequent examinations, any later deviation from which would be viewed with suspicion. It is necessary also in reporting on a water supply for the first time to make several examinations under different weather conditions including an examination after heavy rainfall; thus, a sudden increase in the number of coliform bacilli after rain indicates potential danger as water-borne disease is often associated with flood-water.

A rule originally laid down by Houston has generally been accepted in the past: that a water *from a suspicious source* with *typical B. coli* in 10 c.c. or less should be condemned for drinking purposes. When *typical B. coli* is found in 1 c.c. or less a water may certainly be regarded as unsafe for drinking. On the other hand waters of high purity usually show absence of *B. coli* from 50 c.c. and an efficiently chlorinated water should be free from *B. coli* in 100 c.c.

In the Ministry of Health report it is recommended that piped water supplies (sampled on entering the distribution system) should be classified as follows :—

	Count of *B. coli* in 100 c.c. by the presumptive test.
Class 1. Highly satisfactory	Less than 1
,, 2. Satisfactory	1–2
,, 3. Suspicious	3–10
,, 4. Unsatisfactory	Greater than 10

Throughout the year 50 per cent. of samples of non-chlorinated supplies should fall into Class 1; 80 per cent. should not fall below Class 2; and the remainder should not fall below Class 3. In chlorinated supplies the water should be Class 1. In the case of non-chlorinated waters examined at intervals throughout the year an occasional drop to Class 3 need not be regarded as significant of danger, but if the specimens are frequently in Class 3, or if a specimen falls into Class 4, the water may be regarded as definitely below the safety level. The determination of the type of *B. coli* becomes specially important when there is a fall to Class 3; if this is due to atypical coliform bacilli the result is of less significance from the hygienic standpoint, though it has been found that the appearance of such atypical coliform bacilli in a water supply may be the forerunner of a more serious contamination. In the case of waters from a deep source which are usually highly satisfactory (Class 1), a fall to Class 2 would have to be considered significant. If a chlorinated water drops to Class 2, the question would arise as to the efficiency with which the process is being carried out.

In the examination of samples taken on the consumer's premises if comparison of the water before and after distribution shows an increased number of typical *B. coli* this would suggest some pollution in distribution and would require immediate investigation to detect the source of the contamination. It should be emphasised that in all cases the results of bacteriological examination must be closely correlated with topographical observations by health officers and water engineers on the gathering ground and sources of the water, reservoirs and other water works, and the distribution system generally.

Examination for Streptococci

The type of streptococcus indicative of faecal pollution is the enterococcus (*vide* p. 337). This organism grows in the medium used for the *B. coli* test (*vide supra*) and by itself ferments the lactose but without gas production. Its presence in water can therefore be determined by further examination of the contents of the bottles showing acid or acid and gas fermentation in the *B. coli* test. For this purpose 1 c.c. of the primary culture is mixed with 9 c.c. of sterile water in a stoppered tube which is then kept in a water-bath at 60° C. for 15 minutes. The object is to kill the coliform bacilli, the enterococci remaining alive in virtue of their resistance to heat. A large drop is then transferred with a sterile capillary pipette to a plate of MacConkey's medium and stroked out, or spread on the surface (*vide* p. 167). After incubation the enterococcus, if present, grows in the form of small red colonies. The identity of the organism can be confirmed by subculturing single colonies and examining further.

Another method recommended is to make subcultures on a lactose-tellurite medium from the bottles showing fermentation in the *B. coli* test. This medium contains 1 per cent. peptone, 0·5 per cent. lactose, 0·2 per cent. anhydrous dipotassium hydrogen phosphate, 0·5 per cent. sodium chloride, and is solidified in the usual way with agar; potassium tellurite is added to make a 1 in 15,000 concentration and should be added to the medium after the latter has been sterilised. The enterococcus produces on this medium small bluish-black colonies (see Ministry of Health report cited above).

Examination for B. welchii

50 c.c. of water are added to 100 c.c. of sterile milk in a stoppered bottle of suitable size. The bottle is then heated at 80° C. for fifteen minutes. Sterile liquid paraffin is run on to the surface of the medium to maintain anaerobiosis. On incubation the occurrence of the "stormy-clot" reaction is indicative of the presence of *B. welchii* (*vide* p. 513). To examine larger quantities of water, additional bottles of milk are inoculated each with 50 c.c. of the specimen and treated as above. Varying quantities of water can be tested likewise.

For methods of isolating the typhoid-paratyphoid bacilli and *V. cholerae* from water, see pp. 444, 476.

BACTERIOLOGICAL EXAMINATION OF SEWAGE AND SEWAGE EFFLUENTS

The bacteriological examination of sewage is not of great practical importance, unless in determining the purity of an effluent from a sewage purification process.

The procedure is the same as in water examination; an estimation of the viable bacteria present is made by plating and counting colonies, and the *B. coli* test is carried out as with a specimen of water: much smaller amounts, however, are tested than in the case of water, depending on the likely extent of dilution of the effluent. The numbers of bacteria per c.c. in crude sewage vary greatly—*e.g.* from 1 to 100 millions.

Typhoid and paratyphoid bacilli may be isolated from communal sewage by methods of selective culture. For this purpose the brilliant-green enrichment method (*vide* p. 438), and Wilson and Blair's medium (p. 141), may be recommended.

BACTERIOLOGICAL EXAMINATION OF MILK

In hygiene work the bacteriological examination of milk generally consists in :

(1) An estimation of the number of viable bacteria present in a given quantity.
(2) An estimation of the degree of contamination present from faecal sources—*e.g.* a quantitative *B. coli* test.
(3) The determination of the presence of specific pathogenic organisms—e.g. *B. tuberculosis*.

Since 1936 the *methylene-blue reduction test* has been used as a standard official method in England for gauging milk purity. It depends on the reduction and decolorisation of the dye by the bacteria in the milk, and the rate of reduction affords a measure of the degree of bacterial contamination. This test has therefore been applied as a substitute for the bacterial count in the case of raw milk (*vide* p. 180).

Under the Milk (Special Designations) Regulations, 1936 to 1943, of England, and the corresponding Regulations in Scotland, standard methods for testing milk have been prescribed by the Ministry of Health and the Department of Health for Scotland in official memoranda. These should be consulted for full details of the methods referred to. *See also* **Appendix**—new regulations (1944) for heat-treated (including pasteurised) milk.

Bacteriological Standards.—The following standards are laid down under the Milk (Special Designations) Regulations, 1936 to 1943 :—

England and Wales.

"*Tuberculin-tested*" and "*Accredited*" *milks* when tested by the prescribed method must not decolorise methylene blue within 4½ hours when the sample is taken from 1st May to 31st October, and within 5½ hours when the sample is taken from 1st November to 30th April. These milks must contain no coliform bacillus in 0·01 millilitre.[1]

"*Tuberculin-tested (pasteurised)*" *milk* must not contain more than 30,000 bacteria per millilitre.

"*Pasteurised*" *milk* must not contain more than 100,000 bacteria per millilitre.

Scotland.

"*Certified*" and "*Tuberculin-tested (pasteurised)*" *milk* must not contain more than 30,000 bacteria per millilitre and no coliform bacillus in 0·1 millilitre.

"*Tuberculin-tested*" and "*Standard*" *milks* must not contain more than 200,000 bacteria per millilitre and no coliform bacillus in 0·01 millilitre.

"*Pasteurised*" *milk* must not contain more than 30,000 bacteria per millilitre.

Sampling.—If the milk is contained in sealed bottles, one bottle with unbroken seal would constitute the sample. When the milk is in churns it must be carefully mixed before taking a specimen. This can be done by means of a sterile plunger which is moved up and down several times in the milk. The specimen is then obtained with a sterile dipper and placed in a sterile 4-oz. stoppered or screw-capped bottle. Specimens should be examined as soon as possible, but if there should be any unavoidable delay in testing they should be kept at a temperature of 0° to 5° C..

Technique of estimating the number of viable bacteria.—The medium recommended is that described by G. S. Wilson (*The Bacteriological Grading of Milk*, Medical Research Council, Spec. Rpt. Series, No. 206, 1935, p. 63).

The composition of this medium is as follows :—Yeastrel (Brewers' Food Supply Co. Ltd., Edinburgh) 3 grams, B.D.H. peptone 5 grams, washed shredded agar 15 grams, fresh whole milk 10 c.c., distilled water 1 litre. The yeastrel and peptone are dissolved in distilled water in a steamer and the reaction is adjusted to pH 7·4. The agar is washed and excess water expressed and then it is added along with the milk to the broth. The medium is autoclaved at 15 lbs. pressure for twenty minutes and filtered hot through paper pulp. The pH is adjusted to 7·0 at 50° C. and the medium is tubed in 10 c.c. quantities. These are autoclaved. The final reaction at room temperature should be pH 7·2.

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[1] The millilitre may be taken as equivalent to the cubic centimetre.

A series of dilutions of the milk sample is made up in sterile stoppered bottles with sterile tap water as follows :—

1 in 10	90 c.c. water *plus* 10 c.c. milk
1 in 100	90 c.c. ,, ,, 10 c.c. of the 1 in 10 dilution
1 in 1000	90 c.c. ,, ,, 10 c.c. of the 1 in 100 dilution

Before making these dilutions the specimen should be carefully mixed by repeated inversion of the sample bottle. The dilutions must also be mixed but without vigorous shaking. The pipettes used should be straight-sided and appropriately graduated. For each dilution a separate sterile pipette should be used.

For testing "certified," "tuberculin-tested (pasteurised)" and "pasteurised" milks under the Scottish Milk Order 1 c.c. of the 1 in 100 dilution is plated, duplicate, or preferably triplicate, plates being made ; in examining the other designated milks 1 c.c. of the 1 in 1000 dilution is plated as above. The diluted milk is placed with a sterile pipette in a sterile Petri dish (4-in. diameter) and 10 c.c. of melted agar cooled to 45° C. are added and mixed with the milk.

Under the English milk order, for examining "tuberculin-tested (pasteurised)" and "pasteurised" milks it is prescribed that 1 c.c. of each dilution should be plated. In dealing with a milk of unknown quality this system may also be adopted.

The time between the preparation of the dilutions and the mixing with medium should not exceed fifteen minutes.

After the medium has solidified the plates are incubated for two days at 37° C. in the inverted position.

Under the Scottish order, the number of colonies is counted in each plate and the mean count calculated ; this is multiplied by the dilution and is reported as the "number of viable bacteria per millilitre." The count is made with an artificial illuminant and a hand lens. If the number of colonies in a plate is over 300 a count may be made of those in a given part of the plate and the total is then calculated.

Under the English milk order, a plate showing more than 500 colonies is not to be counted unless the plate made from the next higher dilution shows less than 30. If the plate from the 1 in 1000 dilution has more than 500 colonies it is inferred that there are more than 500,000 bacteria per millilitre of milk. A plate with less than 30 colonies should not be counted unless it is from the 1 in 10 dilution, in which case it is inferred that there are 10 times the number of observed colonies per millilitre of milk.

Under the most favourable conditions a specimen of raw milk may contain at least 500 bacteria per c.c. ; but under bad conditions the numbers may reach even several million per c.c. The standards given on p. 306 indicate the degree of bacterial contamination allowable in the case of the designated milks.

Bacillus Coli Test.—Varying amounts of milk are added to tubes or bottles of bile-salt-lactose medium (as in the *B. coli* water test, p. 296). The range of amounts that require to be tested depends on the likely degree of contamination. In the case of ordinary dairy milk the following series is suggested :—

1·0 c.c. of a 1 in 10 dilution of the milk
,, ,, 1 in 100 ,, ,,
,, ,, 1 in 1000 ,, ,,
,, ,, 1 in 10,000 ,, ,,

The decimal dilutions are prepared in series (*vide supra*).

The smallest amount which yields acid *and gas* is ascertained.

Under the Scottish milk order, for "certified" and "tuberculin-tested (pasteurised)" milks three Durham's fermentation tubes containing 10 millilitres of the above medium are inoculated (by means of a sterile pipette) each with 1 millilitre of the 1 in 10 dilution of the sample and incubated at 37° C. for 48 hours. For the other designated milks, three tubes are inoculated each with 1 c.c. of the 1 in 100 dilution. The tubes are examined for acid and gas production ; the milk is taken to have passed the test if acid and gas are absent from two of the three tubes.

Under the English milk order this test is used in conjunction with the methylene-blue reduction test and the memorandum under the order prescribes the method to be employed. In principle the procedure is the same as that described above.

Methylene-blue reduction test.—Standard methylene-blue tablets must be used. (The names of manufacturers who supply such tablets are furnished by the Ministry of Health.) A standard solution is prepared as follows : one tablet is dissolved in 200 ml. cold sterile glass-distilled water in a sterile flask with a rubber stopper. The solution is then made up to 800 ml. with distilled water and stored in a cool dark place. This solution gives a final concentration of methylene blue of approximately 1/300,000, and should not be used after two months.

Test-tubes conforming to the British Standard Specification 152/16 (6 × ⅝ in.) with an internal diameter of 13·5 mm. (approx.) and a mark indicating 10 ml. are used. They are stoppered with cotton-wool or aluminium caps, and sterilised in a hot-air oven (160° C.—two hours). Rubber stoppers to fit the tubes are also required. These are sterilised in boiling water before use.

A thermostatically-controlled covered water-bath with rack to hold the tubes immersed in the water is required; the water should be at 37°–38° C..

1 ml. straight-sided pipettes are used for measuring the methylene-blue solution (these should conform to a prescribed specification). They are sterilised in the hot-air oven.

The sample is mixed thoroughly, as prior to making the bacterial count (*vide supra*).

The milk is poured, with the usual aseptic precautions, into a test-tube up to the 10 ml. mark, and 1 ml. of methylene-blue solution is carefully added. The tube is closed with a sterile rubber stopper which should be inserted with sterile forceps. It is then inverted slowly once or twice and placed in the water-bath.

The following controls should be put up :—(1) 10 ml. mixed milk *plus* 1 ml. methylene-blue solution, (2) 10 ml. mixed milk *plus* 1 ml. tap water. These control tubes are placed for three minutes in boiling water to destroy the natural reducing system of the milk. Comparison with (1) indicates when decolorisation is beginning and with (2) when it is complete.

The tubes are examined every half-hour, and if no change has occurred on each occasion they are inverted once.

Decolorisation is considered complete when the whole column of milk is decolorised or decolorised up to within 5 mm. of the surface. The time of complete decolorisation is recorded if within the prescribed period (see standards given above).

Examination for Tubercle Bacilli.—The sample is thoroughly mixed and a quantity of 100 c.c. is divided into 50 c.c. amounts and centrifuged for half-an-hour at a minimum speed of 3000 revolutions per minute. The sediment in each tube is suspended in 2·5 c.c. of sterile saline solution. (A microscopic examination of the sediment for tubercle bacilli may be made at this stage, but as shown later is quite unreliable. Before staining, the dried films should be treated with ether for some minutes to remove the fat.) Two guinea-pigs are injected subcutaneously on the inner side of one thigh with the suspended sediment and kept under observation to ascertain whether tuberculous lesions result (*vide* p. 396). One guinea-pig is killed at the end of four weeks and an autopsy carried out ; if it shows no tuberculous lesions the other animal is kept for eight weeks, when it is killed and examined.

An alternative method is to centrifuge 100 c.c. as above and suspend the deposit in 2·5 c.c. of saline. The resultant suspension is injected intramuscularly into the thighs of two guinea-pigs. The animals are kept for five weeks, when both are killed and examined.

Lesions should be examined microscopically for tubercle bacilli to confirm their tuberculous nature. (It has been shown that *B. abortus*, which may occur in cow's milk, produces tubercle-like lesions in guinea-pigs.) It is necessary to inoculate at least two animals from one specimen, as inoculated guinea-pigs may die sometimes from infection with other organisms present in the milk—*e.g.* sporing anaerobic bacilli. This difficulty may be obviated by treating the sediment with antiformin (*vide* p. 400) before injection. Some intercurrent disease—*e.g.* pneumonia, enteritis, etc.—may also cause death before tuberculous lesions have developed and so nullify the test if only one animal is injected.

Some of the cream from the centrifugalised milk may also be used for the inoculation.

The microscopic examination may reveal acid-fast bacilli other than the tubercle bacillus (*vide* p. 405). The absence of tubercle bacilli in films does not exclude their presence in the specimen. The microscopic test, therefore, is not a valid method of demonstrating tubercle bacilli in milk.

The method of *direct cultivation* described on p. 401 can very suitably be applied to unmixed milk taken directly from the cow, but the animal inoculation test is the standard procedure for demonstrating tubercle bacilli in milk samples generally.

Other pathogens in milk.—The methods for demonstrating *B. typhosus* (and *B. paratyphosus*) and *B. diphtheriae* correspond to those used for the isolation of these organisms. For *B. typhosus*, the sediment, after centrifuging, should be plated out on the surface of desoxycholate-citrate medium or Wilson and Blair's medium, and at the same time tubes of brilliant-green peptone water or tetrathionate medium are inoculated as in isolating the organism from faeces (*vide* pp. 436, 438). For *B. diphtheriae*, plates of tellurite medium are inoculated with the sediment (*vide* p. 375).

B. abortus may be demonstrated in milk by inoculating two guinea-pigs as in the test for the tubercle bacillus (*vide supra*). The animals are killed and examined after four and eight weeks respectively, cultures are obtained from the spleen by the appropriate method (*vide* p. 492) and the organism is then identified. An agglutination test with the serum of the inoculated guinea-pig and a suspension of *B. abortus* affords evidence of infection from the milk without the necessity of isolating the organism.

TESTING OF ANTISEPTICS

By " germicide " is meant a substance which destroys micro-organisms. The terms " antiseptic," " bactericide " and " disinfectant " are usually employed as synonyms, though " antiseptic " was originally applied to substances which inhibit bacterial growth. Most substances, however, which inhibit growth are germicides if used in high enough concentration, or if the exposure is sufficiently long.

The " Inhibition coefficient " is the lowest percentage concentration of a particular antiseptic or germicide that will completely inhibit growth in nutrient medium—*e.g.* 0·25 per cent. carbolic acid for *B. typhosus*.

One of the best methods of determining this is to incorporate a series of different concentrations of the antiseptic in 10 c.c.

nutrient agar, pour the medium in plates, and then make a stroke inoculation from a bacterial suspension. This should be prepared in sterile distilled water from a young culture on solid medium, and should show just a faint turbidity to the naked eye. Different organisms can be tested at the same time by making stroke inoculations on each of the various plates. The surface of the medium must be free from condensation fluid.

The plates are incubated for forty-eight hours, when observations can be made.

Instead of solid medium, tubes of broth (carefully standardised as regards pH) or serum (sterile ox serum previously heated at 56° C. for several hours) may be substituted. To a series of such tubes varying concentrations of the antiseptic are added and then each is inoculated with a standard amount of bacterial suspension. The effect is observed after incubation at 37° C. for forty-eight hours, the presence or absence of living organisms being decided by subculturing; one stroke is made from a loopful of each mixture. In this way one agar plate will serve for subcultures from a series of tubes.

In the case of slowly acting bactericidal substances, such as acriflavine and other antiseptic dyes, it is often found that concentrations which have entirely inhibited growth, as shown by their complete transparency, still contain a few living organisms as tested by subculture. The lowest concentration of antiseptic which produces this result (inhibitory concentration) yields a satisfactory index of the potency.

The "INFERIOR LETHAL COEFFICIENT" expresses the concentration of an antiseptic and the time of exposure required to kill non-sporing bacteria—*e.g.* 1 : 60 carbolic acid usually kills haemolytic streptococci in five minutes.

It can be determined by preparing a series of concentrations of the substance in a fixed quantity of diluent (*e.g.* sterile water), adding a constant quantity of bacterial suspension, and transferring at intervals a certain amount of the mixture to some suitable culture medium (*e.g.* broth, or melted agar at a temperature of 45° C., which, after the transfer, is poured in plates).

The quantity of antiseptic carried over to the culture medium must be less than the inhibition coefficient.

The "SUPERIOR LETHAL COEFFICIENT" expresses the concentration of a germicide and the time of exposure required to kill bacterial spores—*e.g.* 4 per cent. potassium permanganate has been found to kill *B. anthracis* spores in twenty minutes.

The "PHENOL COEFFICIENT" expresses the germicidal power of a particular substance as compared with pure phenol.

The method of estimating this coefficient is known as the Rideal-Walker test.[1]

Its chief application is for testing disinfectants composed of coal-tar derivatives which are water-soluble or water-miscible.

Materials Required—

(1) Standard broth, made as follows :—

Lab. Lemco	20 grams
Peptone (Eupepton)[2] . . .	20 ,,
Sodium chloride	10 ,,
Distilled water	1000 c.c.

Boil for thirty minutes, cool, and make up to 1 litre with freshly boiled distilled water. Make neutral at 37° C. to phenol phthalein with normal sodium hydroxide solution. Steam for thirty minutes to precipitate phosphates, and filter whilst hot. Cool, and adjust pH to 7·6 with normal hydrochloric acid. Distribute in 5 c.c. amounts into tubes and sterilise in the autoclave or steamer.

(2) Standard platinum loop of 28 S.W.G. wire, 4 mm. internal diameter, bent almost at a right angle to the wire, so that in the subsequent manipulations the plane of the loop is horizontal.

(3) Phenol. Pure phenol having a crystallising point of 40·5° C. must be used.

(4) Culture of *B. typhosus.* It is of the utmost importance to use always a standard culture of *B. typhosus.*[3]

Subcultures should be made in the standard broth at twenty-four hours' intervals, three times before the test is carried out, and a twenty-four hours' broth culture used for the test proper.

Method of Testing—

(1) Determine beforehand the inhibition coefficient of the particular germicide for the standard strain of *B. typhosus* and make up a series of five graded concentrations, the lowest being slightly greater than the inhibition coefficient (*vide infra*).

The strongest concentration in the series is made up in a sterile stoppered flask with sterile distilled water as the solvent or diluent, and from this the remaining concentrations are prepared by appropriate dilution with sterile water in sterile flasks or tubes. The necessary measurements are made with sterile graduated pipettes.

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[1] For full details of technique, see *Determination of the Rideal-Walker Coefficient of Disinfectants*, British Standards Specification, No. 541, 1934 ; obtainable from British Standards Institution, 28 Victoria Street, London, S.W.1.

[2] Allen & Hanbury.

[3] Obtained from the Curator, National Collection of Type Cultures, Lister Institute, Chelsea Gardens, London, S.W.1 ; the purpose for which the culture is required should be stated.

(2) Make up 100 c.c. of a 5 per cent. stock solution of the pure phenol in sterile distilled water, and from it prepare the following dilutions of phenol :

1 in 95, 1 in 100, 1 in 105, 1 in 110, and 1 in 115.

These dilutions must not be used if more than a week old.

(3) To 5 c.c. (in stoppered sterile test-tubes) of each of the solutions prepared from the germicide to be tested, add with a sterile pipette 0·2 c.c. of the twenty-four hours' broth culture of *B. typhosus* and shake the mixtures. The tubes containing the solutions should be kept during the test in a water-bath at 18° C..

(4) At intervals of 2½ minutes up to 10 minutes remove a large loopful from each mixture, using the standard platinum-wire loop of 4 mm. diameter, and transfer to tubes of 5 c.c. standard broth.

(5) and (6) Carry out with the phenol solutions the same procedure as in (3) and (4).

The bacterial suspension in (3) should be added to the tubes of antiseptic in succession at definite intervals—*e.g.* 30 seconds. The loop-transfers to broth from each tube, after 2½, 5, 7½ and 10 minutes respectively, can then be accurately timed.

(7) Incubate the broth tubes for forty-eight hours, and note those in which growth has occurred.

(8) The coefficient is calculated as follows :—

Divide the figures indicating the degree of dilution of the disinfectant which shows life in 2½ and 5 minutes but no life thereafter *by* that figure indicating the degree of dilution of phenol which shows life in 2½ and 5 minutes but no life thereafter.

The following results illustrate the test :—

Dilution		*Time in Minutes*			
		2½	5	7½	10
Unknown Germicide	1:400	—	—	—	—
	1:500	—	—	—	—
	1:600	+	—	—	—
	1:700	+	+	—	—
	1:800	+	+	+	+
Phenol	1:95	+	—	—	—
	1:100	+	+	—	—
	1:105	+	+	+	—
	1:110	+	+	+	—
	1:115	+	+	+	+

(+ = growth, — = no growth)

$$\text{Phenol coefficient} = \frac{700}{100} = 7{\cdot}0.$$

The Rideal-Walker test compares the action of the antiseptic with that of phenol on *B. typhosus* in distilled water only and does not necessarily give any indication of the disinfecting action under practical conditions where much organic matter is usually present. Accordingly, therefore, the Chick-Martin test has been advocated in which the disinfectant is tested in the presence of organic material, which is the quantity of solid matter present when heat-sterilised liquid faeces containing 10 per cent. of solids is mixed with twice its volume of disinfectant.

The use of faeces in this test is open to several objections, and Garrod has devised a modification of the Chick-Martin test with yeast instead of faeces.

A specification of the standard technique of Garrod's modification has been prepared by a Committee of the British Standards Institution[1] and should be consulted for full details.

The test is somewhat similar to that of the Rideal-Walker test, the difference being that yeast is present and that the dilutions of disinfectant and phenol are allowed to act on the test organism for a fixed time, thirty minutes, instead of varying times. The standard broth and *B. typhosus* culture, however, are the same.

The yeast is made up in suspension in distilled water equivalent to 5 per cent. of dry yeast, and for the test 48 c.c. are added to 2 c.c. of the *B. typhosus* broth culture. 2·5 c.c. of this mixture are added to 2·5 c.c. of dilutions, varying by 10 per cent., of the disinfectant, and of phenol. After thirty minutes, samples are taken exactly as in the Rideal-Walker test. The phenol coefficient is calculated by dividing the mean of the highest concentration of phenol permitting growth and the lowest concentration producing sterility, with the corresponding mean of the disinfectant. Thus, supposing there was no growth with 2·0 per cent. phenol, but growth with 1·8 per cent., the mean is 1·9. Similarly, suppose there was no growth with 0·457 per cent., but growth with 0·411 per cent. of the disinfectant, the mean is 0·434. The result is expressed in the following form:

$$\text{Phenol coefficient} = \frac{1{\cdot}9}{0{\cdot}434} = 4{\cdot}4.\ (\pm\ 10\ \text{per cent.}).$$

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[1] *Modified Technique of the Chick-Martin Test for Disinfectants*, British Standards Specification, No. 808, 1938 ; see footnote (1), p. 313.

TESTING OF SURGICAL CATGUT FOR STERILITY

The following method is prescribed in the Therapeutic Substances Regulations (Therapeutic Substances Act), but an alternative method may be used if approved by the Licensing Authority under the Act, Ministry of Health in England and Department of Health for Scotland.

"A sample of sterilised surgical ligature (suture) shall be taken from each batch consisting of not less than 1 per cent. of the whole quantity of material constituting the batch. The sample shall, when practicable, be the contents of at least one whole container or packet. . . .

the container or packet shall be opened and the sample removed with aseptic precautions ;

after all the adherent fluid has been drained off as completely as possible, the sample shall be placed entire in a test-tube at least 3·5 cm. in diameter and 17·5 cm. in length, and containing 50 mils.[1] of sterile distilled water. This tube shall then be closed by some method which will preclude the access of bacteria, and be placed in an incubator at 37° C. for twenty-four hours ;

after this incubation, the sample shall be aseptically transferred to a similar tube containing a solution of 1 per cent. of sodium thiosulphate and 1 per cent. of crystallised sodium carbonate in distilled water, the tube and solution having been previously sterilised in the autoclave. In this solution the sample shall again be incubated for twenty-four hours at 37° C. ;

after the second incubation the sample shall again be removed aseptically and, without further washing, shall be examined for the presence of living bacteria and their spores.

The sterility tests shall be carried out . . . . by placing the sample in a tube at least 3·5 cm. in diameter and 17·5 cm. in length, containing not less than 50 mils. of a culture medium prepared by dissolving 0·2 per cent. of prepared agar-agar in a nutrient bacteriological broth . . . . the broth may preferably be made by the digestion of meat with trypsin (Douglas's broth or Hartley's modification thereof) . . . . the mixture being sterilised in the autoclave : . . . .

the tubes of culture medium containing the sample shall be incubated at 37° C. for twelve days, and examined daily for the growth of bacteria :

if no such growth is detected during this period, the batch from which the sample was drawn shall be treated as free from living bacteria and their spores, and as having passed the test : . . . ."

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[1] The millilitre may be taken as equivalent to the c.c.

An alternative culture medium which has been used is the cooked-meat medium (described on p. 153) with 15–20 c.c. of broth added to each tube (these being $6 \times \frac{3}{4}$ in.). Before transferring the catgut to the medium from the thiosulphate solution it is placed in a tube of sterile distilled water for a few hours. Meat medium has been found to be particularly valuable for obtaining growths of sporing anaerobic bacilli from catgut, and also serves for the detection of other organisms which may occur as contaminants in catgut.

It should be emphasised that in all the manipulations of the catgut sample in the above specified processes care is required to exclude extraneous contamination, and it is advisable to carry out these manipulations under an inoculating hood, or with the aid of a similar apparatus, to prevent aerial contamination (*vide* p. 167).

COLLECTION AND FORWARDING OF SPECIMENS FOR BACTERIOLOGICAL EXAMINATION

When specimens are forwarded to a laboratory for bacteriological examination they must be placed in appropriate sterile containers to prevent contamination or leakage. The material should be sent to the laboratory as soon as possible after being obtained.

Full particulars should accompany the specimen as this is of great assistance to the bacteriologist when making his report. The container in which the specimen is placed should bear the name of the patient and other relevant details.

For fluids, such as *pus*, *blood for Wassermann and Widal tests*, *cerebro-spinal fluid*, etc., strong glass test-tubes, 5 in. by $\frac{3}{8}$ in., with rubber bungs may be used. The glass tubes are sterilised in the hot-air oven ; the rubber bungs are boiled for five minutes, picked out of the water with forceps, flamed and inserted into the sterile tube.

We recommend the use of a screw-capped bottle known as a *Universal container*, as used in the London County Council Pathological Service, which consists of a strong moulded glass bottle with a flat base and wide mouth, and having a screw-cap. Its size is 3¼ in. high and 1⅛ in. diameter, and the full capacity is 28 c.c. The metal screw-cap is furnished with a red rubber washer 2 mm. thick. These bottles are supplied already cleaned and capped in 1-gross boxes. They are sterilised (with the caps screwed on) by autoclaving and not in the hot-air oven, as the rubber washer will not withstand the temperature. After use the bottles are washed and fitted with new screw-caps; the old ones should be discarded.

The screw-capped universal container has many advantages over the glass tube and rubber bung. It is stronger, cheaper, and is more readily and effectively sterilised. The screw-cap keeps the mouth of the container always sterile, whereas with the tube and bung, dust tends to accumulate at the rim of the tube. The contents cannot leak or become contaminated. The universal container stands quite stable on its base, which is of particular convenience when specimens are taken at the bedside. It can also be used for sputum, faeces, small pieces of tissue, etc..

Faeces.—A small squat bottle of about 2 oz. capacity, or a glass specimen tube 2 in. by 1 in., fitted with a bark cork in which a small metal spoon is fixed, is commonly used. Such containers have the disadvantage that any fermentation of the faeces tends to blow out the cork and cause leakage of the contents. The corks have to be discarded after use. The shoulder on the bottle makes cleaning difficult.

For small quantities of faeces the universal container (*vide supra*) is suitable. A small " spoon " made of tin plate 3⅜ in. × ⅜ in. with one end bent in a small U is employed. These spoons are wrapped in kraft paper and sterilised. For use the spoon is unwrapped, a portion of faeces taken up in the U-shaped end, and the whole dropped into the container and the cap screwed on. Alternatively, a wide-mouth 2 oz. screw-capped jar, known as a " pomade pot," is used, and the faeces taken up in small cardboard spoons (such as are used for ice-cream cartons).

When there is likely to be a delay of some hours before laboratory cultivation can be carried out, two volumes of 30 per cent. neutral glycerol in 0·6 per cent. sodium chloride should be added to the faeces and the whole thoroughly mixed (*vide* p. 436). The solution is buffered to make it alkaline and phenol red is added as an indicator. The fluid should not be used if it becomes acid, which is indicated by a yellow colour. The specimen of faeces is added directly to the container.

The solution is prepared as follows. Make up 30 per cent. glycerol in 0·6 per cent. saline. Add 1 per cent. of anhydrous disodium hydrogen phosphate, and steam to dissolve. Then add 0·02 per cent. phenol red solution (see p. 113) until a purple-pink colour is obtained (about 15 c.c. phenol red solution per litre). Distribute in 6 c.c. amounts in universal containers, a pink bead being added for identification, and sterilise at 5 lbs. The colour should be judged by pouring a small quantity of the solution into a universal container.

Urine.—For small quantities of urine—*e.g.* from cases of enteric, cystitis, etc.—the tube with rubber bung, or universal container (*vide supra*) can be used. For catheter specimens a sterile 6 oz. wide-mouth screw-capped bottle (" honey pot ") is very convenient. The end of the catheter can be placed in the bottle and the urine taken off directly. For larger quantities—*e.g.* 24-hours' specimens—the 20 oz. screw-capped bottles are convenient (*vide* p. 97).

Sputum.—Bottles of 2 oz. capacity, universal containers, or specimen tubes 2 in. × 1 in., fitted with rubber bungs may be used. Where the sputum is not sent by post, screw-capped waxed cardboard cartons of 2 oz. capacity (such as are used for cream and ice-cream) are suitable. The patient expectorates directly into the carton, the wide mouth of which prevents any fouling of the outside, the cap is screwed on, and the name of the patient, etc., written on with an ordinary pencil or grease pencil. After the specimen is examined in the laboratory the carton and contents are burned. Owing to the postal regulations (*vide infra*) the waxed carton cannot be sent through the post. For transmission by post the 2 oz. screw-capped "pomade pot" described above can be used.

Exudates.—Exudates such as pleural fluid coagulate on standing, and the fibrin clot entangles the cells and renders a cell count or centrifuging difficult. The sodium citrate "Biochemical bottle" described below is very suitable for preventing coagulation. The exudate when withdrawn is added directly to the bottle. Clotting does not take place, while the very small quantity of citrate solution present does not affect the accuracy of the count.

Biochemical bottles.[1]—For biochemical examinations of blood it is convenient to place the specimen in a 1 oz. screw-capped bottle (as figured on p. 161) containing the necessary preservative or anti-coagulant and fitted with a perforated cap similar to a blood-culture bottle. The anticoagulant varies with the examination required and the following is a useful range.

Anticoagulant	*Amount*	*Distinguishing mark*	*Use*
Sodium fluoride and thymol	90 mgms. 10 mgms.	Red cap	For blood sugar (venous blood), inorganic phosphorus, uric acid, and non-protein nitrogen tests
Neutral potassium oxalate	40 mgms.	Blue cap	For blood urea, and the majority of blood tests
Sodium citrate	0·3 c.c. of 20 per cent. solution (about 60 mgms.)	Mauve cap	For some blood tests, including animal inoculation; also used in submitting specimens of serous effusions and cyst fluids for general laboratory examination
Sodium fluoride and potassium oxalate	20 mgms. 15 mgms.	Yellow cap	Majority of blood tests

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[1] See McCartney, J. E., and Ayling, T. H., *Lancet*, 1935, i., 1388.

The sodium fluoride and thymol mixture is added to the already sterilised bottle. The potassium oxalate is added in the form of 0·2 c.c. of a 20 per cent. solution, and the fluid is rolled round the bottle to distribute as much as possible on the side; the bottle (uncapped) is then placed in the hot-air oven to evaporate the solution, the object being to have as much potassium oxalate on the side of the bottle and in a thin layer for easy solution on addition of the blood; the sterile cap is then fitted. The sodium citrate solution is placed in the bottle and sterilised by autoclaving (with the cap fitted). The fluoride-oxalate mixture is first made up in solution: sodium fluoride 4 per cent., potassium oxalate 3 per cent.; 0·5 c.c. of the solution is added to each bottle and evaporated as described above.

After the distinguishing colour has been painted on with cellulose paint and dried, the cap is covered with a No. 2 transparent viskap.

For use, the viskap is not removed, but the top wiped with a little spirit. Blood is taken from a vein with needle and syringe, and the needle inserted through viskap and washer and the blood added to the bottle. Not less than 10 c.c. of blood should be taken. Gentle but thorough shaking for three minutes is necessary to ensure solution of the anticoagulant; this is especially necessary in the case of the sodium fluoride.

Blood for blood culture.—Blood can be placed in a sodium citrate "biochemical bottle," or preferably added directly to the medium in the special blood culture bottle described on p. 184.

Swabs.—A swab consists of a piece of aluminium or tinned iron wire, 15 gauge and 6 in. long. One end is made rough for about ½ in. by squeezing it in a small metal vice or cutting edge of pliers. Around the roughened area a thin pledget of absorbent cotton wool is tightly wrapped for about ¾ in.. The wire is placed in a narrow hard glass tube, 5 in. by ½ in. and the top of the tube plugged with cotton wool. Alternatively, and where swabs have to be sent by post, the wire should be 4½ in. long and the top inserted into a bark cork which stoppers the tube. The tube with swab should be sterilised in the autoclave, and not in the hot-air oven, as in the latter the wool may char and give rise to tar-like products which may be inimical to bacteria on the swab (p. 81). These swabs are very useful for taking specimens from :—

(*a*) Throat : in cases of suspected diphtheria, tonsillitis, pulmonary tuberculosis, etc..

(*b*) Wounds, or surgical conditions, *e.g.* fistula, sinus, etc. Some of the purulent material is taken up on the cotton wool.

(*c*) Post-nasal or naso-pharyngeal space : for this purpose the terminal ¾ in. is bent through an angle of 45 degrees, and in use is inserted behind the soft palate. This procedure is useful for suspected meningococcal carriers, and for the early diagnosis of whooping cough (p. 366 and **Appendix,** isolation of *B. pertussis*).

(*d*) Rectum : rectal swabs are very useful in dysentery cases, especially in children.

(*e*) Cervix uteri : in gonorrhoea and puerperal infections. (A longer wire, 9 in., is preferable for these specimens.)

Instead of wire, swabs may be prepared from thin wooden sticks 6½ in. long, which are specially made for the purpose, and are known as " Peerless " wooden applicators. A cotton wool pledget is wrapped round one end as before, and the tube is plugged with cotton wool. They may be used as above under (*a*), (*b*), (*d*) and (*e*), but of course cannot be bent, and cannot be used conveniently with a bark cork.

West's post-nasal swab is described on p. 366.

Where some time may elapse before the swab is examined, and especially where delicate pathogens are concerned, *e.g.* meningococcus or *B. pertussis*, it is advantageous to place about ¼ in. of saline-agar (2 per cent. of agar in 0·85 per cent. sodium chloride solution) at the bottom of the swab tube. This is done before the swabs are sterilised. A bark cork is used as a stopper and the wire pushed through the cork so that the cotton wool is clear of the agar. After the specimen has been taken the swab is inserted into the tube and the wire pushed down until the cotton wool pledget is in contact with the saline-agar.

Tissue.—Small pieces of tissue may be sent in the universal container. Larger pieces of tissue—*e.g.* from *post-mortem* examinations—should be sent in sterile 2 oz., 8 oz., or 1 lb. screw-capped jars according to size.

Material in formalin for sections should be sent in any of the above screw-capped containers.

Postal Regulations.—The Postmaster-General has laid down the following instructions for sending pathological material through the post, and these should be rigorously observed.

> "*Articles sent for Medical Examination or Analysis.*—Deleterious liquids or substances, though otherwise prohibited from transmission by post, may be sent for medical examination or analysis to a recognised Medical Laboratory or Institute, whether or not belonging to a Public Health Authority, or to a qualified Medical Practitioner or Veterinary Surgeon within the United Kingdom, by *letter post, and on no account by parcel post*, under the following condition :—
>
> Any such liquid or substance must be enclosed in a receptacle, hermetically sealed or otherwise securely closed, which receptacle must itself be placed in a strong wooden, leather or metal case in such a way that it cannot shift about, and with a sufficient quantity of some absorbent material (such as saw-dust or cotton-wool) so packed about the receptacle as absolutely to prevent any possible leakage from the package in the event of damage to the receptacle. The packet so made up must be conspicuously marked 'Fragile with care' and bear the words 'Pathological Specimen.'
>
> Any packet of the kind found in the parcel post, or found in the letter post not packed and marked as directed, will be at once stopped and destroyed with all its wrappings and enclosures. Further, any person who sends by post a deleterious liquid or substance for medical examination or analysis otherwise than as provided by these regulations is liable to prosecution.
>
> If receptacles are supplied by a Laboratory or Institute, they should be submitted to the Secretary, General Post Office, in order to ascertain whether they are regarded as complying with the regulations."

The following receptacles have been approved by the Postmaster-General:—

For universal containers, media bottles (Fig. p. 161) and 2 oz. pots, a leatherboard box, internal size 4⅜ in. × 2⅛ in. × 1⅞ in. deep with metal-bound edges and full-depth lid, is used. The glass container is wrapped in a piece of cellulose tissue, 19 in. × 4½ in., and then fits securely in the box which is placed in a shaped gummed envelope having a tag for the postage stamps.

Swabs or cultures in tubes are wrapped in cellulose tissue and placed in hinged metal boxes having rounded corners, size 6¼ in. long, 2½ in. wide, and 1 in. deep. These are placed in stout manilla envelopes which have a tag at the end for the postage stamps.

For the 6 oz. " honey pots " and the 1 lb. jars, a larger piece of cellulose tissue is required, while the leatherboard box is similar in construction to the one mentioned above and large enough to take these receptacles.

Gummed labels printed with the name and address of the laboratory and the information required by the Post Office Regulations, are issued by some laboratories with the postal materials.

PART III

The Pathogenic and Commensal Micro-Organisms (including the Filterable Viruses) and Bacteriological Diagnosis

CHAPTER X

THE PYOGENIC COCCI AND OTHER ORGANISMS ASSOCIATED WITH THE COMMONER SUPPURATIVE CONDITIONS; ORGANISMS ASSOCIATED WITH CONJUNCTIVITIS; PNEUMOCOCCUS

PYOGENIC COCCI

THESE organisms are found in various acute inflammatory and suppurative conditions. They are associated with pus formation, and are therefore designated "pyogenic." The most important are:

Staphylococcus (*pyogenes*) *aureus*, and
Streptococcus pyogenes.

STAPHYLOCOCCUS AUREUS

Morphology.—Spherical cocci arranged in irregular clusters, the individual cells being approximately 0·8–0·9μ in diameter. Single forms and pairs may also be noted.

Staining.—Gram-positive.

Culture.—Aerobe and facultative anaerobe; temperature range—10°–42° C., optimum—about 36° C.; growth occurs on ordinary nutrient media.

Agar stroke—thick, opaque, moist, shiny, "oil-paint"-like growth which develops a characteristic golden- or orange-yellow colour.

Colonies on agar—circular disks, relatively large even after 24 hours (2–3 mm. in diameter), and presenting the same characters as the stroke culture.

Colonies on blood-agar—similar to those on agar but somewhat larger; definite orange-yellow colour; marked zones of haemolysis (*vide infra*).

Gelatin — growth in this medium is associated with liquefaction due to a proteolytic enzyme (gelatinase).

On potato medium, growth is similar to that on agar; on this medium pigmentation is specially marked.

Broth—uniform turbidity, with subsequent orange-coloured deposit.

Milk—acid formation, and coagulation.

Coagulated serum is softened or liquefied.

Haemolysin is produced in culture and is demonstrable in culture-filtrates (*vide infra*).

Various carbohydrates are fermented (with acid but without gas production)—*e.g.* glucose, lactose, saccharose, mannitol.

An enzymic product designated *coagulase* is characteristic of many strains of *S. aureus*; it coagulates blood plasma and can be tested for by adding one drop of a broth culture to citrated rabbit plasma and incubating the mixture at 37° C. for three hours. The occurrence of this product is regarded as indicative of the pathogenicity of the strain (*vide infra*).

Another method of testing (Fisk) which can be recommended is as follows: (1) dilute citrated human plasma 1 : 10 with normal saline; (2) place 0·5 c.c. of diluted plasma in two small test-tubes; (3) to one tube add 5 drops (approx. 0·125 c.c.) of an overnight broth culture or a broth-suspension of an agar culture (opacity equal to that of the broth culture); (4) incubate tubes at 37° C.; (5) examine the tubes after half-an-hour and at intervals for six hours. Clotting usually occurs within an hour. The second tube serves as a control and should show no clotting.

Viability.—The thermal death-point is about 62° C., but some strains are more resistant to heat—*e.g.* withstanding 70° C. for a short time. Laboratory cultures survive for months.

The organism is fairly resistant to drying. It is killed within a few minutes by 2 per cent. phenol.

Staphylococci, like many other Gram-positive organisms, are very sensitive to various organic dyes, *e.g.* crystal violet, brilliant green and the flavine dyes. Thus, brilliant green is bactericidal in a concentration of 1 : 10,000,000, though in the presence of albuminous matter, *e.g.* the serous exudate of a wound, its activity is greatly reduced. The antiseptic action of proflavine, however, is maintained in the presence of serum and under these conditions it is bactericidal to staphylococci in a concentration of 1 : 200,000. These substances are only slightly toxic and proflavine can be used as a wound antiseptic in a 1 : 1000 aqueous solution.

(See also **Appendix**—Penicillin.)

Other Types of Staphylococci

Staphylococcus albus.—Growth is similar to that of *Staphylococcus aureus*, but is unpigmented and white in colour. This type is generally less active than the *aureus* strains in the following properties : liquefaction of gelatin, fermentation of sugars, haemolysin production ; on blood-agar, may or may not show a zone of haemolysis.

It should be noted that colonies of the *albus* type may occur as a variant in cultures of *Staphylococcus aureus*.

Strains of staphylococci corresponding to *S. albus* and occurring as commensals on the skin have sometimes been named *S. epidermidis.*

Staphylococcus citreus.—An uncommon and mainly saprophytic type ; differentiated from others by lemon-yellow colour of growth ; does not generally liquefy gelatin.

Staphylococcus cereus albus and *Staphylococcus cereus flavus* (*Micrococcus cereus*).—Uncommon and mainly saprophytic types ; develop wax-like growths, the former white, the latter yellow. They do not liquefy gelatin.

Staphylococcus ascoformans.—This organism is associated with the condition of " botryomycosis " occurring in equines. In the tissues the cocci are frequently capsulate and, especially in chronic lesions, occur in zoogloea-like masses or clusters. These aggregates may resemble in naked-eye appearance actinomyces " granules " (*vide* p. 528). In culture, capsules are not seen and the organism resembles *S. aureus* in its general characters.

A similar staphylococcal infection, sometimes pathologically resembling actinomycosis, may occur in the udders of cattle and pigs.

Pathogenicity and Occurrence of Staphylococci

Toxins.—Certain strains of the *aureus* and *albus* types are markedly toxigenic. Culture-filtrates are haemolytic (when mixed with blood suspensions), kill leucocytes (when added to a preparation of separated leucocytes), produce necrosis of tissue (when injected into the skin), and exert a rapidly lethal effect on intravenous injection.

Different types of staphylococcal haemolysin can be recognised: the "α" type produces rapid lysis of rabbit and sheep red cells at 37° C.; the "β" type lyses sheep cells, the effect being progressive at room temperature (*e.g.* after the test mixtures of blood suspension and culture-filtrate have been removed from the incubator and allowed to stand overnight at room temperature). It has been pointed out that while human erythrocytes are acted on by the β haemolysin, these cells are only slightly susceptible to the α haemolysin. A third haemolytic factor, designated γ has also been described. These three factors are antigenically distinct. The α haemolysin, however, has been stated to be identical with the necrotoxic and lethal factors referred to above.

A special medium (Walbum's) has been found very suitable for the preparation of staphylococcal toxin; this consists of a meat extract prepared from ox heart to which are added 0·5 per cent. Witte's peptone, 0·2 per cent. potassium dihydrogen phosphate and 0·03 per cent. magnesium sulphate; the pH is adjusted to 6·8. The culture is grown in an atmosphere of 20–25 per cent. carbon dioxide.

Another method is to grow the organism on 0·8 per cent. nutrient agar for forty-eight hours in an atmosphere of 25 per cent. carbon dioxide, and then add to the culture a buffered broth (sugar-free broth containing 4 per cent. peptone with an equal volume of M/15 potassium dihydrogen phosphate solution); the culture is finally incubated for three days in 25 per cent. carbon dioxide as before.

Cases of food-poisoning have been reported which are due to an exotoxin of staphylococci growing in the incriminated article of food, and pronounced local and general toxic effects have been observed in experimental animals under certain conditions after the introduction of staphylococcal culture-filtrates into the stomach. In certain cases milk and milk-products have been responsible for such poisoning, the staphylococcus apparently having originated from udder infection in cows. This *enterotoxin* is different from the other staphylococcal toxins. It is highly thermostable and can resist boiling for a short period.

It has been claimed that strains producing this toxin can be identified by injecting a culture-filtrate intraperitoneally in a kitten, the haemolysins having been first inactivated by heat. The animal develops symptoms of gastro-enteritis—vomiting and diarrhoea—within a few hours. Intravenous injection in a cat may be substituted for this test.

Occurrence.—In localised abscesses, wound suppuration, skin pustules, furuncles and carbuncles, blepharitis, mucous catarrhs, acute osteomyelitis and periostitis, septicaemia, pyaemia, urinary sepsis, etc., *Staphylococcus aureus* is the type usually found in pyogenic lesions, though *albus* strains are sometimes found to be pathogenic.

The staphylococci generally are common commensals of the skin, mouth, nose and throat, especially the *albus* type, and are found in air and dust and on clothing, and on eating and drinking utensils. The *aureus* type is the most virulent ; the others are either non-pathogenic or of relatively low virulence.

The natural *classification of the staphylococci* presents some difficulty. There is now a good deal of evidence that coagulase production is characteristic of the pathogenic strains among which both *aureus* and *albus* forms are represented. These strains are usually mannitol-fermenters and produce haemolysin. It has been suggested that they should be designated *Staphylococcus pyogenes*, while coagulase-negative strains should be classified as *Staphylococcus saprophyticus*. It has also been shown that among the pathogenic strains, at least three types can be recognised by agglutination tests with antisera, whereas non-pathogenic strains cannot be assigned to these serological types and are more heterogeneous.

Immunisation.—In the treatment of chronic or recurrent staphylococcal infections, stock and autogenous vaccines have been extensively applied with somewhat variable success. Staphylococcal toxoid has also been advocated for immunisation in such cases. Staphylococcal antitoxin has been used in the treatment of the more acute and severe infections.

Chemotherapy.—Sulphathiazole, a sulphonamide compound, is of value in the treatment of staphylococcal infections, though sulphanilamide and sulphapyridine have proved relatively ineffective (*vide* p. 333). Highly successful results with penicillin have been recorded in the treatment of severe staphylococcal infections. (See **Appendix**.)

STREPTOCOCCUS PYOGENES

Morphology.—Spherical or oval cocci, 0·7–1μ in diameter, in chains of variable length; involution forms frequent. By relief methods of staining, virulent strains can be shown to have a capsule (p. 211).

Staining.—Gram-positive.

Culture.—Aerobe and facultative anaerobe; temperature range—generally 25°–42° C., optimum—37° C.; grows on ordinary media, but better on blood or serum media.

Agar stroke—growth consists of small circular discrete semi-transparent disks, about 1 mm. in diameter after twenty-four hours' incubation.

On blood-agar—colonies larger than on agar; clear zones develop round the colonies, due to a diffusible haemolysin produced by the organism (*vide infra*).

"Matt" and "glossy" types of colony are observed, the former type being that noted in recently isolated strains from pathological lesions, the latter representing an avirulent variant. A "mucoid" type of colony may also be observed: in virulence it corresponds to the "matt" form.

Broth—growth usually forms as a granular sediment in the culture tube.

Biochemical Reactions.—Tested by growing in Hiss's medium with 1 per cent. of fermentable carbohydrate, and an indicator of acidity (litmus, Andrade's indicator, or neutral red, *vide* p. 128); alternatively on serum-peptone-water-agar slopes containing the particular carbohydrate and an indicator (*vide* p. 119). The reactions are usually as follows :—

Glucose	Lactose	Saccharose	Mannitol	Salicin	Raffinose	Inulin
⊥	⊥	⊥	–	⊥	–	–

(⊥ = acid, no gas ; – = no fermentation.)

Other biological characters of *S. pyogenes* will be referred to in relation to the classification of streptococci generally.

Viability.—The thermal death-point is about 54° C.. The organism can survive for some time in air and dust. Ordinary cultures do not long maintain their viability, but survive best if kept at a low temperature—*e.g.* 0° C.. A convenient method of preserving laboratory cultures for long periods is to grow the organism in cooked-meat medium (*vide* p. 154) and keep the cultures at 0° C.. Cultures which have been rapidly desiccated *in vacuo* and kept *in vacuo* in the dark at low temperatures remain viable for long periods (p. 181). Like staphylococci, *S. pyogenes* is highly sensitive to the antiseptic dyes, *e.g.* proflavine.

Toxic Products.—In addition to the haemolysin, a leucocidin may be present in culture filtrates. Recently isolated virulent strains yield also a diffusible product which rapidly lyses *in vitro* human fibrin (*fibrinolysin*). Another important toxic product is the erythrogenic toxin (*vide* p. 343).

Two antigenically different haemolysins are produced, designated " O " and " S " (Todd). " O " is developed when the organism is growing in serum-free broth and is oxygen-sensitive. " S " is not present in serum-free broth and is not oxygen-sensitive, though very susceptible to heat.

Culture-filtrates also contain a substance capable of producing marked increase in the permeability of tissues (" diffusion factor "). This product is apparently a mucolytic enzyme (" hyaluronidase "). Certain other organisms also yield a similar factor.

Chemotherapy.—Acute infections by *S. pyogenes* usually respond well to treatment with the sulphonamide compounds. These substances seem to act in the blood and tissues by interfering with the metabolism of the streptococcus and by inhibiting its growth so that the natural defences of the body come into full play in destroying the organism. (See **Appendix.**) Infections by *S. pyogenes* are also amenable to penicillin therapy.

CLASSIFICATION OF STREPTOCOCCI AND THE VARIOUS TYPES OF THESE ORGANISMS

The streptococci constitute a somewhat heterogeneous group, and, besides the common pathogenic strains, e.g. *S. pyogenes*, include types which are normal inhabitants of the mouth, throat and intestine.

These organisms have been broadly classified in medicine according to the *appearance of growths on blood-agar*. Thus, in the case of *Streptococcus pyogenes*, wide zones of haemolysis develop round the colonies (*vide supra*), and this is a characteristic feature. The designation " haemolytic " or " β (*beta*) " has been applied to this type of streptococcus.

The most reliable method of identifying a haemolytic streptococcus is to grow it in 20 per cent. serum broth for about six hours and then to add varying amounts (*e.g.* 0·1 to 1 c.c.) of the culture to 0·5 c.c. of a 5 per cent. suspension of ox or horse red blood corpuscles. The mixtures are incubated at 37° C. for one and a half hours, when haemolysis can be observed.

Certain varieties occurring as commensals in the mouth and throat are practically non-haemolytic and their colonies on blood-agar show a greenish coloration, due probably to peroxide formation and the resultant production of methaemoglobin. (Some degree of partial haemolysis may also be noted round the colonies.) The colour change is most marked when the organism is growing on a medium containing heated blood (*e.g.* " chocolate agar ") and is probably due to an oxidation product of haematin. Streptococci producing such changes on blood media are designated *Streptococcus viridans*, or the " α (*alpha*) " type. Varieties which in the past have been named *S. salivarius* and *S. mitis* are of this type.

It has been found that the *viridans* form may under certain conditions be derived as a variant from a haemolytic organism. This is probably a weakly haemolytic derivative, and if reducing sugar is removed from blood-agar, or if the organism is cultivated anaerobically, the variant exhibits the typical β haemolysis.

A third group found as commensals in the mouth, throat and bowel produces no obvious change in blood media, and is sometimes designated the "γ (*gamma*)" type.

Morphological differences are of little significance in classification, though the faecal streptococci ("enterococcus") are usually oval in shape, or even lanceolate like a pneumococcus (*q.v.*), and occur in pairs or short chains.

Streptococci producing a mucoid capsule have been described—"*Streptococcus mucosus.*"—On blood-agar it yields slimy colonies with dark-green coloration of the blood; it has been noted in various suppurative conditions—*e.g.* otitis media. "*Streptococcus epidemicus.*"—This organism is of the haemolytic type but produces a mucoid capsule and its growth on medium is likewise mucoid. It has been reported in milk-borne outbreaks of sore throat.

Fermentative reactions have been extensively utilised for the differentiation of streptococci, but these cannot be regarded as sufficiently "fixed" characters for accurate classification. Certain biochemical tests, however, are of some significance in the general grouping of the streptococci. Thus, the faecal streptococci are generally mannitol- and aesculin-fermenters: the salivary types usually ferment raffinose. It has been pointed out that among the haemolytic streptococci those of human origin ferment trehalose but not sorbitol, whereas strains fermenting sorbitol but not trehalose are of animal origin (*vide infra*).

Streptococci differ considerably in their *resistance to heat*, and this has been utilised as a basis of classification. Thus, the faecal strains (enterococcus) are able to withstand 60° C. for thirty minutes whereas this temperature kills the haemolytic streptococci and also the common mouth types.

Other differential features.—The enterococcus is usually able to grow between 10° and 45° C. (*cf. S. pyogenes*) and has considerable viability in culture. It can be cultivated also in the presence of 6·5 per cent. sodium chloride and at a pH of 9·6, *i.e.* conditions under which other streptococci cannot grow. The final pH of a growth in 1 per cent. glucose broth is 4·0–4·2, whereas *S. pyogenes* yields a final pH of 5·2 (*vide* p. 117).

The enterococcus is also capable of growing on media containing bile-salt (*e.g.* MacConkey's) which is inhibitory to other types. A certain proportion of strains of this organism differ from other streptococci in liquefying gelatin. These are sometimes designated *S. liquefaciens.*

The *Streptococcus lactis* found in soured milk is biologically similar in some respects to the enterococcus, but probably represents a separate serological group (*vide infra*). It does not grow at pH 9·6 and is not resistant to heat at 60° C.. It differs also from the enterococcus in the absence of saccharose fermentation.

Anaerobic (or *microaerophile*) *streptococci* have been described. They correspond in their general biological characters to the aerobic forms. Microaerophile haemolytic strains have been found to be associated particularly with spreading necrotic infections of the skin. Some anaerobic streptococci are non-haemolytic and foetid gas formation in culture is a common characteristic of these. Such organisms have been observed in septic lesions and in gangrenous conditions of mucous membranes. They are sometimes present in puerperal sepsis and may produce a blood infection. They have been found normally on mucous surfaces.

The following table summarises the main differential features of the three medically important groups of streptococci.

	Pyogenic streptococci	*Mouth and throat streptococci*	*Enterococcus (faecal streptococci)*
Morphology	Chains of moderate length; cocci spheroidal	Chains of pronounced or moderate length; cocci often elongated	Usually in pairs but may occur in short chains; cocci oval or lanceolate
Type as regards effect on blood (*vide supra*)	β	α or γ	γ
Mannitol-fermentation	Usually −	−	+
Heat resistance (*vide supra*)	−	−	+
Inhibition of growth by bile-salt	+	+	−

SEROLOGICAL CLASSIFICATION OF THE STREPTOCOCCI

Lancefield's groups.[1]—By means of precipitation reactions with antisera, Lancefield has distinguished a number of groups of streptococci, and it is now possible to correlate this grouping to some extent with that based on other characters including pathogenicity to man and animals. These precipitation reactions depend on group-specific antigens or haptens, certain of which have been defined as carbohydrates.

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[1] See Lancefield, R. C., *J. Exper. Med.*, 1933, **57**, 571-95; Hare, R., and Colebrook, L., *J. Path. Bact.*, 1934, **39**, 429-42; Lancefield, R. C., and Hare, R., *J. Exper. Med.*, 1935, **61**, 335-49.

Group A.—β-haemolytic on blood-agar; produce O and S haemolysins (p. 333): ferment trehalose but not sorbitol (*vide supra*). The strains are of human origin, and are associated with infections of the human subject, often of a severe nature. This group includes all Griffith's serological types (*vide infra*) with four exceptions, types 7, 16, 20 and 21. It is the most important group from the medical standpoint, and strains belonging to it must be regarded as pathogenic or potentially pathogenic. Organisms of this group correspond to the classical type described on p. 332 as *S. pyogenes*.

Group B.—Includes both haemolytic and non-haemolytic strains as judged by lysis on blood-agar; the β-haemolytic strains produce the S haemolysin, but this property is a less frequent feature of Group B than Group A. Found in bovine mastitis and milk. Some strains from the human vagina and the throat also belong to Group B. This group is rarely pathogenic to the human subject but has been recorded in a few cases of puerperal infection assuming a pyaemic form. Streptococci of Group B correspond to the organism designated *S. agalactiae* (p. 346).

Group C.—β-haemolytic; produce the S haemolysin. This group is found mainly in animals—e.g. *Streptococcus equi* of Strangles in horses, but may also occur in human infections, including puerperal sepsis.

Group D.—Originally described as β-haemolytic on blood-agar. Such strains were isolated from human faeces and the vagina, and from cheese, and their relationship to the enterococcus was recognised. The S haemolysin has been demonstrated in this group. It now seems likely that all strains of the enterococcus, irrespective of their haemolytic action, belong to Group D.

Group F.—β-haemolytic on blood-agar, and the S haemolysin has been demonstrated. Colonies are minute and growth is slow. Strains have been found mainly in infection of the throat and upper respiratory passages. It has been noted that Group F infection may be associated with nephritis.

Group G.—Produce the O and S haemolysins like Group A. The majority of strains have been found as commensals in the human subject, though the group includes pathogenic representatives.

Groups designated *E*, *H* and *K* have been defined. These may occur in the human subject as commensals and occasionally as pathogens, mainly in the upper respiratory passages. *Groups L* and *M* have been found mainly in dogs. *Streptococcus lactis* represents a further group designated *N*. This group is quite non-haemolytic.

It will thus be seen that of the strains infecting the human subject the great majority belong to Group A. This differentiation is of particular value in relation to puerperal fever in which, with a few exceptions, the infecting strains of streptococci belong to Group A, whereas the haemolytic streptococci which may occur normally in the birth canal belong to other groups.

When β-haemolytic streptococci are found either in the parturient woman or attendants it is important to determine the group. Therefore, in examining a streptococcus the first point to determine is whether it produces a filterable haemolysin in fluid culture; its particular group is then ascertained.

The precipitating serum is prepared by injecting rabbits with known strains of the particular groups. Broth cultures are centrifuged and the deposited organisms killed with 0·2 per cent. formalin, and diluted with this fluid so as to contain 2000 million per c.c. The rabbits are given a series of four daily intravenous injections of 1 c.c. followed by four days' rest. When the appropriate group serum and a bacterial extract (*vide infra*) containing the specific soluble substance are mixed a profuse flocculation occurs within a few minutes, easily visible to the naked eye.

It is important to secure a potent serum, as the precipitate is soluble in excess of antigen (bacterial extract). A weak serum will give a feeble precipitate which is at once re-dissolved. The antigen is at first tested undiluted; if no reaction occurs it should be re-tested in dilutions of 1 in 3 and 1 in 9. Alternatively, the three concentrations can be tested simultaneously. With a weak serum, a reaction may only be seen with the highest dilution of antigen.

The *technique* of the test is as follows :—

Pick off a haemolytic colony from the plate and inoculate 2 c.c. of 20 per cent. horse-serum broth and incubate for six hours or overnight. Add one volume (0·5 c.c. is a convenient amount) of the culture to one volume of 5 per cent. suspension of horse red corpuscles in saline and incubate the mixture at 37° C. for two hours. Haemolysis is usually complete within thirty minutes. If the streptococcus is haemolytic, an antigenic extract is prepared to ascertain to which group it belongs.

From the serum-broth culture inoculate 50 c.c. of 0·05 per cent. glucose broth and incubate overnight. Centrifuge the culture and pour off the supernatant fluid so that the sediment of organisms is as dry as possible.

Add 1 c.c. of N/20 HCl and two drops of 0·2 per cent. Congo red solution and re-suspend the sediment. Add one or two drops of N/1 HCl until a slate-blue colour is obtained. Transfer the contents to a conical centrifuge tube to which is attached a small piece of adhesive plaster on which the name or number of the culture should be written. Immerse the centrifuge tube in boiling water for ten minutes. The colour of the material should now change to pink. Cool the tube under running water. Centrifuge and remove carefully the supernatant fluid, the sediment being discarded. Add one drop of phenol red solution and neutralise cautiously with N/1 NaOH and re-centrifuge. The resultant clear supernatant fluid constitutes the antigenic extract.

The method of carrying out the precipitation test is to add the different concentrations of the extract (*vide supra*) to an equal volume of undiluted serum in narrow tubes so that the extract is superimposed on the serum. Precipitation is first observed at the interface of the two reagents usually within five minutes, but becoming more pronounced when the tubes are incubated for two hours and then allowed to stand for a time at room temperature.

If no reaction occurs with Group A serum, the procedure should be repeated with Groups C and G sera, and if necessary with other group sera.

An alternative method which can be recommended is that in which formamide is used in the preparation of the antigenic extract (*Fuller's method*). Add 0·1 c.c. of formamide to the bacterial sediment from 5 c.c. of culture (*vide supra*), shake the tube thoroughly and place in an oil bath at 150° C. for fifteen minutes. (The metal cup of a thermos flask makes a simple oil bath, olive oil or liquid paraffin being used.) Cool and add 0·25 c.c. of acid-alcohol—95 parts of absolute alcohol with 5 parts of 2N hydrochloric acid (1 part of concentrated acid to 4 parts of water). Mix and centrifuge to separate the resulting precipitate. Pipette off the supernatant fluid into a small tube and add 0·5 c.c. acetone. Shake the tube, centrifuge and discard the supernatant fluid. Add 1 c.c. of saline and a drop of phenol red indicator and neutralise the solution with a trace of sodium carbonate. This constitutes the antigen for the test.

In order to conserve serum, the test may be made in a capillary tube 2 in. long. This form of tube is best obtained by purchasing the capillary tubes used for vaccine lymph and cutting them in two.

Make a mark on the capillary tube, about ½ in. from the end, draw up antigen to this mark and expel the drop on a paraffined slide (made by dipping a microscope slide into melted paraffin wax and allowing it to cool in the vertical position). Place a similar volume of Group A serum beside it, and mix with a fine straight platinum wire. Hold the slide at an angle, touch a drop of the mixture of antigen and serum with the capillary tube held horizontally, when the fluid will enter the tube. Seal one end of the tube in the flame. Place the tube in a piece of plasticine, or in a block of wood with a number of holes made with a No. 42 Morse drill.

Macroscopic precipitation should occur within a few minutes and certainly within half-an-hour in the incubator. If left overnight cross reactions may occur. The procedure can be repeated with different dilutions of the antigen (*vide supra*) and if no reaction occurs the same test is carried out with antisera for Groups C and G.

Griffith's types.—By agglutination and agglutinin-absorption tests streptococci have been found to be exceedingly heterogeneous. Thus, Griffith separated thirty serological types of haemolytic streptococci which he considered of epidemiological significance. Of these types the commonest are 1, 2, 3, 4, 6, and 8. All but types 7, 16, 20 and 21 belong to Group A. The antisera, which are prepared by injecting rabbits with killed cultures of known type-strains, usually show cross reactions and have to be purified by absorption with other types. The agglutination test is carried out on a slide as described on p. 254, the agglutination being visible in a few minutes to the naked eye. The organisms are grown in serum-broth overnight, the supernatant fluid is removed, and the resultant dense suspension of streptococci used directly. Frequently the streptococcus is "rough," the resultant suspension showing visible clumps of organisms, and the test cannot be carried out. The streptococcus has then to be repeatedly sub-cultured until a homogeneous suspension is obtained.

It is convenient to do a preliminary test with pooled sera, each representing five types. Having found agglutination with a certain pool of sera, the streptococcus is tested with the individual type-sera comprising the pool.

The typing of streptococci by Griffith's method is useful in studying outbreaks of scarlet fever or sore throat, in which all the cases show the same type of streptococcus. If a carrier or suspected source of infection yields the same type it is strong presumptive evidence of the origin of the outbreak. It has been shown that the complications of scarlet fever are due, in the majority of cases, to a re-infection with a different type of streptococcus.

THE PATHOGENICITY AND OCCURRENCE OF STREPTOCOCCI

The pathogenic effects of the streptococci depend on the virulence of the particular strain and the susceptibility of the host. Thus, a strain of low virulence may produce only a localised inflammation, whereas strains of high virulence may be associated with spreading inflammation often followed by septicaemia.

Haemolytic streptococci (β type) are found in the following conditions :—

Wound suppuration ; localised abscesses ; spreading inflammations—*e.g.* erysipelas, cellulitis, lymphangitis, lymphadenitis ; septicaemia (*e.g.* puerperal septicaemia) and pyaemia ; suppurative otitis and mastoiditis ; meningitis, usually secondary to some other lesion—*e.g.* otitis ; ulcerative endocarditis ; inflammation of the fauces, tonsils, pharynx and larynx—*e.g.* acute follicular tonsillitis, the angina of scarlet fever (*vide infra*) ; broncho-pneumonia secondary to influenza, measles, etc..

In septic lesions, mixed staphylococcal and streptococcal infections are not infrequent.

Streptococci may also occur as secondary invaders —*e.g.* in diphtheria, etc..

In the great majority of human infections the β streptococci belong to Lancefield's Group A ; the occurrence of other groups has been referred to on p. 338.

It should be noted that virulent haemolytic streptococci may be found in the throats of healthy persons ; from such carriers may originate cases and outbreaks of various forms of streptococcal disease—*e.g.* tonsillitis, scarlet fever, puerperal infection, wound sepsis, etc..

The frequent association of the *acute rheumatic state* with *antecedent* infection of the throat or upper respiratory passages by haemolytic streptococci is now well recognised, but in the past there has been no evidence that this condition is due to infection of the blood by these organisms or that the joint and cardiac lesions are the results of localisation of streptococci in the affected tissues. Recently, however, haemolytic streptococci have been demonstrated, by cultivation in a rich digest broth (p. 102), to be present in the vegetations on the heart valves in fatal cases of acute rheumatism with endocarditis. It has been claimed that the essential clinical and pathological manifestations of the rheumatic state represent an allergic reaction of the tissues to the products of these organisms in specially predisposed individuals.

Non-haemolytic types (α and γ) may occur as pathogens in lesions of a less acute form than those produced by the haemolytic varieties—*e.g.* cases of tonsillitis, otitis media, dental abscesses, broncho-pneumonia, cholecystitis. Subacute infective endocarditis is usually due to the *viridans* type of streptococcus.

SCARLATINA

It has been long recognised that haemolytic streptococci are constantly present in the throat in early cases of scarlet fever, and also that the majority of the complications of this disease are streptococcal. In 1923 G. F. and G. H. Dick produced clinical scarlet fever by inoculating the throat of a volunteer with a culture of a haemolytic streptococcus isolated from the disease; and later they demonstrated that in filtrates of fluid cultures of scarlatinal streptococci there is present a toxin which in suitable dilutions produces a local erythema when injected intradermally into persons susceptible to the disease, while convalescents generally show no reaction. The test, now designated the *Dick reaction*, is made by injecting intradermally into the skin of one forearm 0·2 c.c. of a dilution (*e.g.* 1 in 1000) of filtrate from a broth culture, while into the other forearm is similarly injected as a control a like amount of the same diluted filtrate which has previously been heated at 100° C. for at least one hour to destroy the toxin present. In a positive reactor a red erythematous patch appears in six to twelve hours, at the site of injection of the toxin. The redness remains for about twenty-four hours and then slowly fades. The control injection—*i.e.* heated toxin—should produce only a transient redness just after inoculation, and no erythema when readings of the test are made. For the reaction to be recorded as positive the diameter of the erythematous area should be at least 1 cm.; a typical positive usually shows an area 2–3 cm. in diameter. Pseudo-reactions, in which both toxin and control injections produce an erythematous reaction, may occasionally occur in adults, but such results are relatively uncommon in children. A positive Dick reaction indicates that the subject is sensitive to the streptococcal toxin, and that there is an absence of natural or acquired immunity. The reaction is positive in the early stages of scarlet fever, being visible over the redness of the rash, but becomes less intense as the disease progresses, and is usually negative after the third or fourth week of the disease. It may be stated briefly that a positive Dick reaction signifies susceptibility to scarlet fever, and an absence of immunity to the streptococcus toxin. Up to the present it has been found difficult to standardise streptococcus (Dick) toxin accurately as laboratory animals do not normally react to this product. By tests in known susceptible persons the toxin can be standardised in terms of the "skin-test" dose (S.T.D.)—*i.e.* the minimum amount which, injected into the skin, produces in twenty-four hours an erythematous reaction of at least 1 cm. diameter.

The toxin is prepared by growing a strain of scarlatinal streptococcus in Hartley's broth for forty-eight hours at 37° C.. The culture is then centrifuged at high speed and the supernatant fluid decanted and passed through a tested earthenware (Berkefeld) or Seitz filter. The filtrate constitutes the "toxin." Phenol (0·5 per cent.) is added as a preservative, and for use the toxin is diluted with normal saline or preferably the buffer solution used for the Schick test reagents (*vide* p. 383) so that 0·2 c.c. of the dilution contains one skin-test dose.

It has been shown that serum from a convalescent scarlet fever patient (0·2 c.c. of a 1 in 10 dilution), when injected intradermally in an early case of scarlet fever, causes a blanching or extinction of the rash around the site of injection. This is termed the *Schultz-Charlton reaction*, and shows that convalescent serum contains neutralising substances for the scarlet fever toxin. A similar result is obtained with antitoxic serum from an immunised animal (*vide infra*). This test may be employed clinically for diagnosis in doubtful cases of scarlet fever.

Active immunisation in susceptible individuals (*i.e.* Dick-positive) is carried out by injecting saline dilutions of toxin made up so that a certain volume —*e.g.* 1 c.c.—contains a given number of "skin-test" doses. Successive and increasing doses are given at weekly or fortnightly intervals, ranging from 500 to 80,000 "skin-test" doses. This treatment usually causes the Dick reaction to become negative.

By the immunisation of horses with streptococcus (Dick) toxin, an antitoxic serum is obtained which is used for therapeutic purposes.

The antitoxin is concentrated and refined in the same manner as diphtheria antitoxin (*q.v.*). It is difficult to standardise accurately owing to the insusceptibility of laboratory animals to the toxin.

Standardisation has been attained, however, by neutralisation tests in Dick-positive reactors, mixtures of serum and toxin being injected intradermally. Thus, in the U.S.A. the antitoxic unit adopted is that amount of serum which just neutralises 50 skin-test doses of toxin. 6000 units are recommended as an initial therapeutic dose.

The average dose of the refined product is 20 c.c., and if given intravenously reduces very considerably the toxic manifestations and incidence of complications, and shortens the stay in hospital. It should be noted that this antitoxin is effective in other conditions due to haemolytic streptococci such as erysipelas, cellulitis, etc., and it is not necessary to have special antisera prepared for streptococci associated with these diseases.

The scarlatinal streptococci belong to Lancefield's Group A (*vide* p. 338). They can be assigned by agglutination reactions to the various types defined by Griffith (*vide* p. 341). The most frequent types are Nos. 1, 2, 3 and 4. There is no serological distinction between scarlatinal strains and haemolytic streptococci from other lesions in the human subject.

THE OCCURRENCE OF STREPTOCOCCI IN DISEASES OF THE DOMESTICATED ANIMALS

Streptococci are relatively infrequent in suppurative lesions of horses, sheep and swine, but may be met with more commonly in cattle. Conditions regarded as due to these organisms are mastitis of cattle, contagious pleuropneumonia of horses, omphalophlebitis of new-born animals with metastases in joints, septicaemia of chickens (*S. gallinarum*).

Bovine mastitis.—The most prevalent organism in this condition is the streptococcus, and the type commonly found, especially in the chronic form of the disease, has been designated *S. agalactiae* : characterised as a long-chained streptococcus producing a flocculent type of growth in broth, acidifying and coagulating milk within forty-eight hours, fermenting glucose, lactose and saccharose but not acting on mannitol ; the strains vary in their action on blood-agar, being of the α, β or γ form. The final pH of growths in glucose broth is 4·2–4·6 (*vide* pp. 117, 336). These organisms exhibit the property of hydrolysing sodium hippurate. This is tested by growing the organism in broth containing 1 per cent. sodium hippurate, and incubating for five days. To 1 c.c. of the supernatant fluid of the culture is added 0·2–0·4 c.c. 12 per cent. ferric chloride solution containing 2·5 c.c. conc. hydrochloric acid per litre, the tube being immediately shaken.

A precipitate which remains insoluble is taken to indicate the presence of benzoate, which in turn signifies hydrolysis of the hippurate. Three main serological types distinguished by agglutination reactions have been described. *S. agalactiae* corresponds to Lancefield's Group B (p. 338).

Some strains of streptococci isolated from mastitis are indistinguishable from the haemolytic forms found in pyogenic lesions of the human subject (Group A), and it has been assumed that such organisms are of human origin. The milk of cows suffering from such streptococcal infection may give rise to outbreaks of sore throat or scarlatina in the human subject, the type of streptococcus being actively haemolytic. This organism has sometimes been designated *S. epidemicus* (*vide supra*).

Bacteriological diagnosis of bovine mastitis.—This is usually carried out by plating centrifuged deposits from milk samples on a medium which is relatively selective for streptococci, viz. crystal-violet-blood-agar. It consists of 1000 c.c. Lemco agar (pH 7·4) with 2 c.c. 0·1 per cent. crystal violet, 50 c.c. defibrinated ox blood and 1 gm. aesculin.[1] Many of the other organisms present in milk are inhibited by the crystal violet and those which are dye-resistant usually produce black colonies in the presence of aesculin and so can be differentiated at sight from streptococci. Deep plating in the medium may be an advantage for detecting haemolytic colonies. Pure cultures of the streptococci present are obtained from single colonies and these are then identified by further tests (*vide supra*).

Strangles of horses is due to a haemolytic streptococcus (*S. equi*), which can be assigned to Lancefield's Group C (*vide* p. 338).

Reference to streptococci occurring in animals has also been made on p. 338 in relation to Lancefield's serological grouping of the streptococci.

MICROCOCCUS TETRAGENUS (*Gaffkya tetragena*)

Morphology.—Spherical cocci in tetrads, about 0·7μ in diameter, and capsulated when growing in the tissues.

Staining.—Gram-positive.

Culture.—Aerobe and facultative anaerobe ; optimum temperature—37° C. ; grows well on ordinary media.

Agar—growth resembles *Staphylococcus albus.*

Gelatin—not liquefied.

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[1] See Edwards, S. J., *J. Compar. Path.*, 1933, **46**, 211.

Occurrence.—Suppuration in region of mouth and neck—*e.g.* dental abscess, cervical adenitis, pulmonary abscess, etc..

Cultures of *M. tetragenus* are pathogenic to the mouse, producing a generalised infection. Thus, it may sometimes be isolated from mixed cultures by injecting this animal.

OTHER ORGANISMS ASSOCIATED WITH THE COMMONER SUPPURATIVE CONDITIONS

BACILLUS PYOCYANEUS (*Pseudomonas aeruginosa*)

Morphology.—Straight rods; 1·5 to 3μ by 0·5μ; motile with one to three terminal flagella; non-sporing.

Staining.—Gram-negative.

Culture.—Aerobe and facultative anaerobe; temperature range—18°–43° C., optimum—30°–37° C.; grows on ordinary media.

Agar—stroke-inoculation produces an abundant, moist, greenish-blue fluorescent growth; the pigment ("pyocyanine"), on which the colour depends, also diffuses through the medium. Pigment is most abundantly produced at room temperature.

A similar but purely saprophytic organism is *B. fluorescens*, which is commonly found in water and soil.

Gelatin—liquefied, with a greenish-blue coloration.

Potato—growth is similar to that on agar but the pigment alters, as a result of oxidation, to a brownish colour.

Occurrence.—Suppurating wounds, usually in mixed infection with pyogenic cocci, otitis media, etc.. This organism may occur as a commensal in the bowel of man and animals. In young subjects it may give rise to enteritis and may even produce a general infection.

BACILLUS PROTEUS (*Proteus*)

Morphology.—Straight rods about same size as *B. pyocyaneus*; pleomorphic; motile with lateral flagella; non-sporing.

Staining.—Gram-negative.

Culture.—Grows aerobically on ordinary media. Optimum temperature about 25° C., but grows well at 37° C.. A single stroke-inoculation on agar produces a moist, translucent, greyish-white growth, *which tends to spread all over the available surface of the medium*—hence the designation " spreader " often applied to this organism (*vide* p. 352). Gelatin is usually liquefied. This property, however, may be lost after the organism has been artificially cultivated for some time. Coagulated serum is liquefied by some strains.

Some non-liquefiers of gelatin are now assigned to this group, *e.g.* Morgan's bacillus (p. 457).

A non-motile variant type of *B. proteus* may be met with which does not spread on culture medium ; it corresponds to the " O " variants of other motile bacteria (*vide* p. 41).

Biochemical Reactions.—*B. proteus* includes different biological types which vary somewhat in fermentative reactions :

Glucose	Lactose	Dulcitol	Saccharose	Mannitol	Maltose	Indole formation	Serum liquefaction
+	−	−	±	−	±	±	±

(+under carbohydrates signifies acid and gas production)

H_2S is formed, and urea is actively converted into ammonia.

Occurrence. — Septic infections — *e.g.* suppurating wounds, urinary sepsis, otitis media, etc.—usually along with other pyogenic organisms. It may be present in faeces, particularly in children, and occurs frequently as a saprophyte in decomposing organic matter. It has been reported as the causative organism in cases of acute bacterial food-poisoning. Organisms assigned to the *B. proteus* group have been described in ozaena (e.g. *Coccobacillus foetidus ozaenae* of Perez)—but this organism does not liquefy gelatin. It is doubtful if these are primary causal organisms in this condition.

In typhus fever the blood serum agglutinates a *B. proteus* type (" X19 ") and to a lesser degree another type (" X2 "), originally isolated from the urine in this disease. The reaction with " X19 " is utilised as a diagnostic test (" Weil-Felix reaction "), but the organism has no aetiological relationship to the disease (*vide* p. 559). *B. proteus* strains of the "X19" type are serologically different from other strains. This type also differs from other strains in the fermentation of salicin. The agglutinin responsible for the Weil-Felix reaction is of the "O" type (*vide* p. 41).

The Weil-Felix reaction has been explained on the basis that *B. proteus* "X19" and the organism of typhus fever possess a common specific polysaccharide, which enters into the constitution of their antigens (*vide* p. 561).

Another type designated "Kingsbury" is agglutinated by the serum of patients suffering from a typhus-like disease in Malaya, and is used in the diagnosis of this condition (*vide* p. 562). Salicin and maltose fermentation are absent (as compared with the "X19" type). This type may not liquefy gelatin.

BACTERIOLOGICAL DIAGNOSIS OF PYOGENIC INFECTIONS

(See also **Appendix**—Bacteriological Examination of Wounds.)

COLLECTION OF SPECIMENS AND MICROSCOPIC EXAMINATION

Films are made on microscope slides from the pus or inflammatory exudate, dried and fixed by heat. The films should, if possible, be made directly from the lesion, a sterile platinum loop or sterile capillary pipette (p. 164) being used to collect the exudate and transfer it to the slides. For transmission to the laboratory the material is placed (by means of a pipette) in a suitable sterile container (*vide* p. 317). If only a minute amount of exudate can be obtained for transmission to the laboratory, a convenient method is to collect it in sterile capillary tubes, the ends of which are then sealed in a flame. The exudate runs into the tube by capillarity when one end is dipped in it. Swabs (p. 321) are very convenient for collecting specimens of exudate, but drying of the material may occur, if there is much delay in transmission to the laboratory. In the case of *ulcers*, *sinuses*, etc., exudate should be taken, if possible, with a sterile platinum loop, and films (and also cultures) are made at once, or the exudate is collected in capillary tubes or on swabs.

In the case of *pleural and peritoneal fluids*, the fluid should be withdrawn into a 1 oz. screw-capped "biochemical bottle" containing four drops of 20 per cent. sodium citrate solution (p. 320). This is to avoid coagulation, which renders cytological and bacteriological examination difficult. The material is centrifuged and films are made from the deposit.

Urine should be drawn, with aseptic precautions, by means of a catheter smeared with a sterile lubricant, *e.g.* glycerin-jelly. No antiseptics are to be used. The specimen is passed directly into a sterile stoppered bottle or 6 oz. screw-capped pot (p. 319), and should be submitted for investigation without any delay. In the female this procedure is absolutely necessary to avoid contamination. In the male, however, a fairly satisfactory specimen may be obtained by cleansing the urinary meatus and, after a portion of the urine has been voided, collecting a sample directly into a sterile bottle. The urine is centrifuged, and films are made from the deposit. After the films have been dried and fixed they should be gently washed in water to remove crystalline deposit.

Sputum should be expectorated directly into a suitable sterile container as described on p. 319. Films are made as in the case of pus.

Films are stained (1) by Gram's method ; (2) with Löffler's methylene blue.

CULTIVATION

Successive stroke-inoculations are made on blood-agar in Petri dishes or sloped in tubes or bottles (*vide* p. 161). A proportion of the growth will be represented by separate colonies, and the colony characters of the organisms can be recognised. Films are also made from colonies and stained by Gram's method. In this way the organism present can generally be identified. If a mixed growth results, single colonies can be picked off on to blood-agar slopes, so that pure cultures are available for any further examination required.

If *septicaemia* or *pyaemia* is suspected, blood culture is carried out (*vide* p. 182).

A difficulty frequently encountered in the bacteriological examination of pus, particularly from infected wounds, is the overgrowth of pyogenic cocci, *e.g.* streptococci, by other organisms present, such as *B. coli*, *B. proteus* and *B. pyocyaneus*. This may be obviated by making use of the selective bacteriostatic effect of potassium tellurite incorporated in the medium used for the primary culture. Thus, if microscopic examination of the pus reveals large numbers of Gram-negative bacilli, tellurite may be added to the agar used for plating, in a 1 in 50,000 concentration (0·2 c.c. of a 1 per cent. solution in 100 c.c. agar). *B. coli* and *B. pyocyaneus* are inhibited and the cocci, if present, can be more easily isolated. *B. proteus* is sometimes resistant to this concentration, but a 1 in 20,000 concentration (0·5 c.c. of a 1 per cent. solution in 100 c.c. agar) may serve to inhibit the spread of this organism on plates, which often makes the isolation of other organisms difficult if not impossible.

The use of 6 per cent. agar has proved a most convenient method of preventing spread of *B. proteus* on plates.

ORGANISMS SPECIFICALLY ASSOCIATED WITH CONJUNCTIVITIS

KOCH-WEEKS BACILLUS (*Haemophilus conjunctivitidis*)

Associated with acute contagious ophthalmia.

Morphology.—Short slender rods about 1–1·5μ in length; intracellular position in polymorph leucocytes of the inflammatory exudate is characteristic.

Staining.—Gram-negative.

Culture.—Does not grow on ordinary media but like

B. influenzae (*q.v.*), to which it is closely similar, can be cultivated on media containing blood; in its growth requirements it is in fact identical with the influenza bacillus. On blood-agar, growth develops in the form of minute dewdrop-like colonies.

DIPLO-BACILLUS OF MORAX (*Haemophilus lacunatus*)

Associated with sub-acute or chronic conjunctivitis.

Morphology. — Rod-shaped organism measuring about 2μ by 1μ, in pairs end to end; non-motile.

Staining.—Gram-negative.

Culture.—Aerobe; requires blood or serum for growth; optimum temperature is about 37° C., and no growth occurs at room temperature; on solidified serum, growth produces liquefaction, and colonies develop "pits" or "lacunae" on the surface of the medium.

Diplo-bacillus liquefaciens (Petit) is morphologically similar to the bacillus of Morax, but grows well on ordinary nutrient media at 20°–37° C.. It liquefies coagulated serum, but, unlike the other, grows in gelatin at 22° C. and liquefies it. It is associated with conjunctivitis in which there may be primary involvement of the cornea.

DIAGNOSIS

Films should be made from a loopful of conjunctival exudate. Stain with dilute carbol fuchsin, or by Gram's method. Make cultures on blood-agar. Pure cultures should be obtained from single colonies and the biological characters of the particular organism determined.

Other organisms found in conjunctivitis are: the gonococcus, pneumococcus, meningococcus, staphylococci, streptococci, organisms of *B. coli* group. *Staphylococcus albus* and diphtheroid bacilli—e.g. *B. xerosis* (*q.v.*)—are frequent normal inhabitants of the conjunctival sac.

PNEUMOCOCCUS (*Diplococcus pneumoniae*)

The causative organism of acute lobar pneumonia.

Morphology.—The typical appearance is that of a lanceolate or oval coccus in pairs with the rounded ends together; it is about 1μ in its long diameter; shows a thick capsule, which appears as an unstained zone round the organism, unless positively stained by special methods (*vide* p. 210). In culture it is not so typical, being less lanceolate and more rounded; the capsule is not so evident, and the cocci may occur in chains.

Staining.—Gram-positive.

Culture.—Aerobe; optimum temperature, about 37° C.; does not grow below 25° C.; grows on ordinary media, but best in the presence of blood or serum.

The addition of glucose (*e.g.* 0·1 per cent.) to culture media promotes the growth of the pneumococcus. It should be noted in the preparation of a broth for the cultivation of this organism that it may be inhibited by an oxidised constituent of the peptone (H. D. Wright). This difficulty can be obviated by adding the peptone to the medium before heating so that it is later subjected to the reducing action of the meat infusion (p. 100). Commercial peptones may also contain metallic impurities which are responsible for inhibition effects in the cultivation of the pneumococcus (*vide* p. 100).

It is sometimes advantageous to grow the organism in an atmosphere of 5 per cent. carbon dioxide.

On agar—growth consists of small (about 1 mm. diameter), delicate, semi-transparent, dewdrop-like colonies, which tend to remain discrete—*i.e.* like colonies of streptococci.

In broth—growth shows at first a uniform turbidity, but later forms a granular deposit in the tube.

On blood-agar—non-haemolytic, or only partially lytic; growth produces the same greenish coloration as *Streptococcus viridans*. Colonies are at first flat and smooth, but later may develop elevated margins and concentric ridges (likened to a draughtsman).

Under anaerobic conditions the pneumococcus may be more actively haemolytic. The haemolysin is readily inactivated by oxidation.

Cultures may show transformation of the colonies from the typical smooth (S) type to the rough (R) form ; this variation is associated with loss of capsule formation, absence of type-specificity (*vide infra*) and loss of virulence.

Biochemical Reactions.—Ferments various carbohydrates (*e.g.* glucose, lactose, saccharose) and differs from most strains of streptococci in its fermentation of inulin. Hiss's serum water, containing 0·1 per cent. of peptone, is a convenient medium for these tests. The agar medium used for testing the biochemical reactions of the gonococcus also gives good results (*vide* p. 131).

Bile solubility.—It is sharply differentiated from the streptococci by its solubility in bile. This test consists in adding 1 part of sterile ox bile or 1 part of a sterilised 10 per cent. solution of sodium taurocholate in normal saline to 10 parts of a broth culture. The test may also be carried out by using a 10 per cent. solution of sodium desoxycholate, 0·1 c.c. being added to 5 c.c. of a broth culture which should not be more acid than pH 6·8 ; this method gives very satisfactory results, lysis occurring within fifteen minutes.

Viability.—The thermal death-point is about 52° C.. The ordinary laboratory cultures lose viability rapidly and require to be subcultured at short intervals (*e.g.* about fourteen days). Cultures survive for a longer period in a semi-solid agar containing blood. If it is desired to maintain cultures over a considerable time without subculturing, they should be preserved by the method of rapid drying *in vacuo* (p. 181).

Experimental Inoculation.—Pathological material containing the pneumococcus (*e.g.* pneumonic sputum) or a virulent culture, injected subcutaneously into rabbits or mice, produces a rapidly developing septicaemia, fatal in twenty-four to forty-eight hours ; at autopsy, typical capsulated diplococci are present in large number in the heart blood. The virulence for animals rapidly decreases if the organism is grown on media without blood. By drying the spleen of an infected mouse in a desiccator, the virulence of the organisms present may be maintained for a month or more. (The spleen should be removed immediately after death.)

Serological Types.—In recent years thirty-three types have been recognised, differing in their reactions with specific antisera, though cross-reactions have indicated that this classification is still incomplete (see **Appendix**). These types have been designated numerically I, II, III, etc. Types I and II are the so-called " epidemic " types, being responsible as a rule for over 50 per cent. of cases of acute lobar pneumonia. Type III is recognised also by its cultural characters ; it is the " *Pneumococcus mucosus*," and produces raised mucoid or slimy colonies.

At one time only Types I, II and III were serologically defined, all other strains being classified as " Group IV." It was noted that the pneumococci occurring normally as mouth and throat commensals mostly belonged to this group.

The determination of the particular type of pneumococcus is important if type-specific antisera are used for therapeutic purposes.

The *identification of the type* is carried out by using the agglutination test with specific sera, or by the direct method, in which capsule-swelling is the criterion of specific interaction between the organism and the appropriate serum.

Agglutination Method.—For rapidity of obtaining a suspension of the organism advantage is taken of the extreme susceptibility of the mouse. The animal is inoculated intraperitoneally with about 0·5 c.c. of the sputum and when it appears ill or moribund is killed and the contents of the peritoneal cavity are washed out and suspended in 1 c.c. of saline. This suspension of organisms is usually dense enough for the test, but it may be centrifuged and re-suspended in a smaller quantity of saline if necessary. A direct agglutination test is done by the slide method as described on p. 254. Lederle's typing sera (*vide infra*) are used, tests being made first with the sera pooled in groups. A drop of bacterial suspension is placed on the slide and a drop of pooled serum " A " (*vide infra*) placed beside it and the two mixed. If the result is positive visible clumping will be observed easily with a lens or the low power of the microscope. If pooled serum " A " gives a negative result, the other pooled sera are used in turn until a positive result is obtained. When a pooled serum gives a positive result, the pneumococcal suspension is then tested with the type-sera comprising the pool until the actual type is identified. It is usually possible to obtain a result at once by this method. Sometimes the suspension from the peritoneal cavity is granular so that agglutination cannot be observed ; in this case cultures are made on blood-agar and in glucose-broth, and the latter culture is used for typing as above. The blood-agar culture serves to identify the organism present as a pneumococcus or otherwise, if no result is obtained from the typing with the glucose-broth culture.

Direct Method of Typing the Pneumococcus (*Neufeld reaction*).—It has been shown that when the pneumococcus, present in sputum from a case of pneumonia, is acted on by a specific serum its capsule becomes swollen and the organism exhibits a clear refractile capsular zone. This reaction, which occurs within three minutes, has been utilised as a direct method of typing. The procedure is as follows : an emulsion of the sputum is prepared in physiological saline solution and separate drops of this emulsion, deposited on slides, are mixed with double the amount of the respective undiluted type-antisera (rabbit). (A similar drop mixed with saline serves as a control.) If the sputum is not tenacious, flecks taken up with a wire loop may be used instead of the drops of emulsified material. The mixtures are covered with No. 1 cover-slips and examined with an oil-immersion lens, the substage condenser being suitably lowered and the diaphragm reduced in aperture. The enlargement of the capsules in the presence of the particular type-serum can readily be observed within a few minutes. It is emphasised that the sharpness of outline of the capsule is more significant than its enlargement. Some workers prefer to add a drop of methylene-blue solution to the preparations ; the expanded capsules remain unstained and present a ground-glass appearance. The sputum should be examined within one hour after expectoration.

This is the method which has been generally used for determining the type of pneumococcus in clinical cases. A set of type-sera for the various types are required, and to simplify the determination a preliminary test is done with sera pooled in groups, *e.g.* as follows : " A " for Types I, II and VII, " B " for Types III, IV, V, VI and VIII, " C " for Types

IX, XII, XIV, XV, XVII and XXXIII, and three further mixtures comprising the other types. After the organism has been assigned to one of these groups, it can be tested with the individual sera of the group. Sera for typing pneumococci can be obtained from the Lederle Laboratories Inc., New York, through Chas. F. Thackray Ltd., 10 Park Street, Leeds, 1.

Specific Substances of the Pneumococcus.—Type-specificity is dependent on polysaccharide haptens (*vide* p. 35) contained in the capsule of the organism. Loss of the capsule-forming property and R transformation of the colonies (*vide supra*) are associated with absence of the specific carbohydrate and of type-specificity and also loss of virulence.

It has been shown that the specific polysaccharides of Types I, II and III are " excreted " in the urine in the majority of cases of pneumonia due to these types, and can be demonstrated by a precipitin reaction with type-sera. In the early stages of the disease the polysaccharide is present in the urine only in a proportion of cases, but these have a graver prognosis than cases in which it is not found.

Occurrence.—In lobar pneumonia the pneumococcus is present often in considerable number in the consolidated areas, and can easily be detected in the sputum. It can be demonstrated in the blood by blood culture, and also occurs in the pathological complications of pneumonia—*e.g.* pleurisy, empyema, endocarditis, pericarditis, meningitis, arthritis, etc..

The pneumococcus is found in broncho-pneumonia, simple catarrhal conditions of the throat and respiratory tract, conjunctivitis, otitis media, primary meningitis, primary peritonitis.

As noted above, this organism may be present normally in the mouth and throat secretions.

In this country Types I and II are together responsible for the majority of cases of acute lobar pneumonia—*e.g.* 50 per cent. or over. Type III is less frequent—*e.g.* 10 per cent. or less. The other types considered collectively (*Group IV*) are found in a varying proportion of cases, usually under 30 per cent. The relative prevalence of types varies in different localities and at different times. The mortality statistics of pneumonia indicate that Type III is the most virulent, and that Type II is more virulent than Type I. Types I and II are, however, more invasive than the others, as shown by their prevalence in primary meningitis and peritonitis, and the occurrence of Type III and *Group IV* in otitis media and conjunctivitis. *Group IV* comprises a large proportion of the strains found in primary broncho-pneumonia in young subjects.

Therapeutic Antisera.—Prior to the introduction of sulphonamide therapy monovalent antisera for the various types of pneumococcus were extensively used with successful results, especially in cases of pneumonia due to Types I, II, V, VII, VIII and XIV. Such sera are concentrated and refined, which allows a large dose of antibodies to be administered intravenously without inconvenience and with little risk of serious shock due to the foreign protein. The sera are standardised according to their power of protecting mice against virulent pneumococci and the " Felton unit " is that amount of serum which protects a mouse against 1,000,000 lethal doses of pneumococcus culture. Repeated doses of 10,000–40,000 such units are administered.

Chemotherapy.—Pneumococcal infection has also proved amenable to treatment with sulphonamide compounds, *e.g.* sulphapyridine (" M. and B. 693 "). These substances seem to act on the organism *in vivo* in the same way as they affect the haemolytic streptococci (*q.v.*).

The use of serum-therapy has diminished as a result of the introduction of sulphonamide therapy, though a combination of the two methods in certain cases may yield better effects than chemotherapy or serum-therapy alone.

Successful results have also been reported with penicillin therapy.

DIAGNOSIS

A specimen of sputum is obtained (*vide* p. 319), and films are stained by Gram's method. Cultures are made by successive strokes on a blood-agar plate. (It is an advantage to incubate in an atmosphere of 5 per cent. carbon dioxide.) The characteristic colonies can be recognised among the other organisms that constantly develop from sputum, and are picked off on to blood-agar slopes for the purpose of isolation. The fermentation of inulin and bile-solubility are determined with the pure cultures obtained.

The occurrence of septicaemia with characteristic diplococci in the heart blood on inoculation of a mouse with the sputum or the culture isolated is conclusive proof of the identity of the organism present, even if the diplococci noted in the specimen or cultures are not entirely typical.

The serological type can be determined by the methods described above.

In a case of acute pneumonia blood culture is of value in determining the presence or absence of septicaemia which is of prognostic significance.

Repeated blood-cultures may be of value in ascertaining whether the blood infection is progressive or not. In this case it is advisable to add a given volume (*e.g.* 1 c.c.) or varying quantities to melted agar, so as to obtain a count of the number of colonies.

See **Appendix**—Use of *p*-aminobenzoic acid in medium.

CHAPTER XI

MENINGOCOCCUS; GRAM-NEGATIVE COCCI OCCURRING IN THE NOSE AND THROAT; GONOCOCCUS; VEILLONELLA

MENINGOCOCCUS (*Neisseria intracellularis*)

CAUSATIVE organism of epidemic cerebro-spinal meningitis; may also produce septicaemia (sometimes chronic).

Morphology.—Oval diplococci with opposed surfaces flattened or concave; sometimes in tetrads; cocci are about 0·8 – 1μ in diameter; the long axes of the cocci in pairs are parallel, not in line as in the case of the pneumococcus (*q.v.*). In cerebro-spinal fluid the intracellular position in polymorph leucocytes is characteristic. In culture the characteristic shape and arrangement seen in the spinal fluid may be lost, and involution forms may be present.

Staining.—Gram-negative.

Culture.—Aerobic conditions are necessary for satisfactory growth; temperature range is 25°–42° C., and the optimum is about 37° C.; requires blood or serum for growth; optimum pH is 7·0–7·4. A specially suitable medium is a nutrient agar prepared from a digest basis, and containing 5 per cent. blood added to the melted agar at 90° C. (*vide* p. 134).

Colonies on serum-agar—after twenty-four hours are small, greyish, transparent, circular disks about 2 mm. in diameter—*i.e.* larger than colonies of streptococci; later the centre of the colony becomes more opaque and raised, while the periphery remains thin and transparent; the borders may become crenated. While this is the common type of colony, considerable variation in the appearances may be noted.

Colonies on blood-agar are like those on serum-agar but somewhat larger; they are smooth, grey and semi-transparent; no haemolysis occurs.

Biochemical Reactions.—Can be tested by growing on peptone-water-agar slopes containing 5 per cent. serum, 1 per cent. of the particular sugar, and an indicator (*vide* pp. 119, 121). The medium described on p. 131 is recommended for these tests.

Ferments glucose and maltose with acid production, but has no action on lactose, saccharose, or inulin.

Viability.—When first cultivated artificially the meningococcus tends to die out quickly in culture—*e.g.* within two or three days. In culture it persists best at incubator temperature. Cultures remain viable for a longer time on an egg medium. For maintaining cultures 1 per cent. agar in Hartley's broth *plus* 20 per cent. serum gives good results. When desiccated under ordinary atmospheric conditions dies within two hours, but cultures can be preserved by rapid drying *in vacuo* (p. 181).

Serological Types.—Gordon recognised two main types (Types I and II) differentiated by agglutination and agglutinin-absorption reactions with antisera, and representing over 80 per cent. of all cases occurring in this country. Other less frequent serological types (*e.g.* III and IV) were also identified.

These types are not so sharply differentiated as the types of the pneumococcus, and Griffith classified strains into two serological groups, one of which (*Group I*) includes Gordon's Types I and III, and the other (*Group II*), Types II and IV. He also showed that the antigenic structure of individual strains may be complex and made up of multiple constituents.

The majority of cases of cerebro-spinal meningitis at the present time are due to Group I; whereas most of the strains found in the naso-pharynx of persons who have not been in contact with cases belong to Group II (*vide infra*). It has been concluded that Group II is of lower pathogenicity than Group I.

Agglutinating antisera are prepared by immunising young rabbits with successive and graded doses of dead cultures (*vide* p. 259) injected intravenously. The initial dose should be 500–1000 million organisms. The serum of the immunised animal may agglutinate the homologous organism in dilutions up to 1 in 400 or 1 in 800. In carrying out the agglutination test, the general method described in Chapter VIII may be followed ; the tubes should be placed in a water-bath at 55° C. for twenty-four hours, when the results can be read.

Animal Inoculation.—In general it has been found difficult to establish an active infection in laboratory animals by inoculation with cultures. Intraperitoneal injection in mice of even small doses of the meningococcus *suspended in a solution of gastric mucin* brings about a rapidly fatal general infection.

Toxin.—The meningococcus is actively toxigenic, and potent toxic products have been obtained from cultures. The toxin has been classified as an endotoxin. Some evidence, however, has been brought forward that it is antitoxinogenic but this has been questioned.

Therapeutic Antisera.—Polyvalent antisera and also monovalent sera for individual serological groups and types have been used—administered by spinal injection and intravenously. Such sera are *antibacterial* in their properties (*vide* p. 36), but it has been claimed that their efficacy depends also on antitoxin (*vide supra*). Serum treatment has been superseded by sulphonamide therapy.

Chemotherapy.—Meningococcal infections respond well to treatment with the sulphonamide compounds, *e.g.* sulphapyridine (" M. and B. 693 "), sulphathiazole and sulphadiazine (*vide* p. 359 and **Appendix**).

DIAGNOSIS

In the early stages of cerebro-spinal meningitis the organisms are present usually in considerable numbers in the cerebro-spinal fluid, and can be recognised by microscopic examination. At a later stage they may be scanty and even apparently absent.

Lumbar Puncture.—The specimen of fluid is obtained by lumbar puncture with a special nickel or platinum-iridium needle fitted with a stilette. The patient is anaesthetised if necessary, and placed on the right side with the knees drawn up and the left shoulder thrown forward. The skin over the lumbar region, the operator's hands, and the needle are sterilised by appropriate methods, and a sterile container (*vide* p. 317) should be ready for collection of the fluid. The puncture is made in the middle line between the 3rd and 4th, or 4th and 5th lumbar vertebrae, the needle being inserted, with the stilette in position, in a forward and slightly upward direction. In the adult it is introduced to a depth of about 5–6 cm. The stilette is withdrawn, and the fluid is allowed to flow into the container. In a case of cerebro-spinal meningitis the spinal fluid is under pressure, and turbid in appearance due to the large number of pus cells present.

In the laboratory the fluid is centrifuged, and films are made from the sediment and stained by (*a*) methylene blue, (*b*) Gram's method. Cultures should also be made on one of the blood or serum media referred to above. Films are made from the resulting colonies or growth and stained by Gram's method. The colony characters should be ascertained, and subcultures for further tests are obtained by picking off single colonies on to blood- or serum-agar slopes. The biochemical reactions should be tested and the serological group is identified by agglutination tests with the appropriate antisera.

For clinical diagnosis the microscopic examination is generally sufficient — *i.e.* if Gram-negative, intracellular diplococci with the characteristic shape of the meningococcus are observed. While the meningococcus is present in large number usually at an early stage of the illness, it may become relatively scanty in the cerebro-spinal fluid at a later stage and even undetectable by microscopic methods. The same change may occur within 24 hours of administering sulphonamide drugs. The meningococcus may be demonstrated by cultural methods when difficult to find by microscopic examination. A method which sometimes facilitates the cultivation of the organism is to add an equal volume of glucose-broth to the cerebro-spinal fluid and incubate the mixture for eighteen hours;

thereafter sub-inoculations are made on a solid medium as described above.

In cases of suspected meningococcal septicaemia blood culture should be carried out.

See **Appendix**—*p*-aminobenzoic acid in culture medium.

Other Meningeal Infections.—In routine investigations of meningitis, other causal organisms must be considered—*e.g.* pyogenic cocci, pneumococcus, tubercle bacillus, etc. If no organisms are detectable in films stained by methylene blue or Gram's method, a Ziehl-Neelsen preparation should be examined for tubercle bacilli (*vide* p. 400). In tuberculosis the cell exudate is mainly lymphocytic at an early stage of the illness, as compared with the polymorph exudate in meningococcal and pneumococcal meningitis. (In meningococcal meningitis at a later stage of the illness there may be a fair number of lymphocytes and mononuclear cells in the spinal fluid, though this is rarely found in early cases ; further, in tuberculous meningitis polymorph leucocytes may sometimes be fairly numerous.) In young children, acute meningitis may be caused by a haemophilic bacillus, similar in characters to *B. influenzae* (*q.v.*). This organism usually develops elongated filaments, and these are seen among the polymorph leucocytes of the exudate. It should be noted that the virus of poliomyelitis may produce an inflammatory condition of the meninges, indicated by the presence of a leucocytic exudate in the spinal fluid. In such cases no visible organisms are present. In acute lymphocytic chorio-meningitis (*vide* p. 616) there is a lymphocytic exudate and absence of cultivable organisms.

DIAGNOSIS OF MENINGOCOCCUS CARRIERS

During an epidemic of cerebro-spinal meningitis healthy contacts may become carriers, and the meningococcus is found in the naso-pharynx. The organism may also be found in the naso-pharynx of healthy persons who have not been in contact with cases and during non-epidemic times. The recognition of carriers has been utilised in the past for controlling the spread of the disease. The practical utility, however, of this measure is doubtful. During an epidemic the carrier-rate among contacts may be very high and the segregation of carriers may present great difficulty. Moreover, it has been found where outbreaks occur in groups of persons living together, *e.g.* troops, that spread can be checked by increasing the space per person in sleeping quarters and by improved ventilation and environmental hygiene. In a limited community when a case of cerebro-spinal meningitis occurs it may be of some value to swab the naso-pharynx of immediate contacts and to isolate any carriers detected in this way. The detection of a carrier depends on culturing the meningococcus from the naso-pharyngeal secretion.

Cultures should not be made within an hour after a meal, or within twenty-four hours after the application of antiseptics to the throat. The specimen is best obtained by means of a swab (*vide* p. 322), with a longer wire-holder than the usual throat swab, and with the terminal $\frac{3}{4}$-in., carrying the cotton-wool pledget, bent through an angle of about forty-five degrees. The swab with wire-holder is enclosed in a stoppered test-tube of sufficient width to admit the bent end. It is necessary that cultures should be made immediately after swabbing and the medium incubated at once, owing to the feeble viability of the organism apart from the body.

The tongue is depressed, and the swab is passed behind the soft palate and introduced into both posterior nares. Before withdrawal, the swab is also rubbed over the posterior wall of the naso-pharynx. The swab must be introduced and removed from the mouth without touching the tongue.

West's Post-Nasal Swab is specially adapted for the purpose; it consists of a curved tube containing a flexible wire with a wool pledget as in the case of a throat swab. The tube is introduced into the mouth, and by means of its curve can be passed up behind the palate; from this end the swab is protruded into the naso-pharynx. It can then be retracted, and the tube is withdrawn from the mouth.

The swab is rubbed *at once* over a small area at the edge of a serum- or blood-agar plate already prepared and warmed to 37° C., and then successive stroke inoculations are made on the remainder of the plate by means of a platinum loop, the loop being charged several times from the area inoculated directly with the swab. It is advisable to use agar containing a digest basis. The plate must be incubated without delay.

When it is impossible to make an immediate culture a convenient method of maintaining the viability of the meningococcus is to place in the foot of the swab-tube a small amount of blood-agar so that the swab, when returned to the tube, is kept in contact with the medium. It is stated that under these conditions the meningococcus may remain viable for 24 hours (Downie). In the laboratory the swab is used to inoculate a blood-agar plate, and the swab-tube is also incubated.

Suspected colonies are examined by means of Gram-stained films, and subcultures from single colonies are made on serum- or blood-agar slopes.

The resulting pure cultures are then available for identification. The differentiation of other Gram-negative throat diplococci from the meningococcus is considered below. The absence of growth at room temperature, the inability to grow on ordinary meat-infusion medium, the sugar reactions, and the readiness with which the growths emulsify in saline, are important features in the identification of the meningococcus.

The final identification is carried out by means of a polyvalent agglutinating anti-meningococcus serum or antisera to the different groups or types.

THE GRAM-NEGATIVE DIPLOCOCCI OCCURRING AS COMMENSALS IN THE NOSE AND THROAT

DIPLOCOCCUS CATARRHALIS (*Neisseria catarrhalis*)

A frequent commensal in the throat and nose, and often regarded as pathogenic in catarrhal inflammations of the respiratory tract.

Morphology and Staining.—Practically identical with the meningococcus. In some strains the cocci are relatively large.

Culture.—Grows on ordinary media without serum and at room temperature; the colonies may be larger than those of the meningococcus, especially when fully grown, and are thicker and more opaque. The colony characters, however, may vary considerably, and both "smooth" and "rough" forms are observed. The organism exhibits no fermentative properties (*vide infra*). Cultures when emulsified in saline tend to be autoagglutinable.

D. catarrhalis is not agglutinated by meningococcus antisera.

DIPLOCOCCUS PHARYNGIS FLAVUS TYPES
(e.g. *Neisseria flava*)

The *morphology* of these organisms is like that of *D. catarrhalis*, and they grow on ordinary media at room temperature. Cultures develop, after forty-eight hours, greenish-yellow or greenish-grey colours.

Young colonies may simulate closely those of the meningococcus.

Biochemical reactions, which vary according to the type, are shown in the Table on p. 369.

They are not agglutinated specifically by meningococcus antisera.

DIPLOCOCCUS PHARYNGIS SICCUS (*Neisseria sicca*)

Resembles *D. catarrhalis*, but its colonies are markedly dry, tough and adherent to the medium. It seems possible that this organism is not a separate species, but a " rough " variant of some other member of the group.

Biochemical Reactions.—See Table.

DIPLOCOCCUS MUCOSUS

Differs from the other members of the group in being capsulate and producing mucoid colonies. This type also may represent a variant of one of the other members of the group.

DIPLOCOCCUS CRASSUS

Resembles *D. catarrhalis*, but shows marked variation in its staining by Gram's method, some individual cells staining Gram-positive, others Gram-negative. The colonies are small and rather like those of the streptococci. Growth occurs at room temperature. It is open to question whether this organism should be assigned to the *Neisseria* group.

Biochemical Reactions.—See Table.

Classification.—It should be noted that there is much uncertainty regarding the biological classification of this group of organisms, and the taxonomic significance to be attached to colony characters, pigmentation, and fermentation of different carbohydrates is doubtful. The group, however, can be broadly divided into two subgroups: (1) characterised by complete absence of fermentative properties, *e.g.* the classical *D. catarrhalis*, and (2) possessing such properties, *e.g.* the *D. pharyngis* types.

Neisseria flavescens

This organism has been described as the causative organism in a group of cases of meningitis in America. It resembles the meningococcus in morphology, but on blood-agar produces golden-yellow colonies. It does not ferment carbohydrates.

FERMENTATIVE REACTIONS OF GRAM-NEGATIVE DIPLOCOCCI

	Glucose	Maltose	Lactose	Saccharose
Meningococcus . .	$\perp$	$\perp$	—	—
Gonococcus . .	$\perp$	—	—	—
D. catarrhalis . .	—	—	—	—
D. pharyngis flavus types	$\perp$	$\underline{\perp}$	—	[illegible]
D. pharyngis siccus .	$\perp$	$\perp$	—	$\perp$
D. crassus . . .	$\perp$	$\perp$	$\perp$	$\perp$

($\perp$ = acid ; $\underline{\perp}$ = variation in reaction among different types)

GONOCOCCUS (*Neisseria gonorrhoeae*)

The causative organism of gonorrhoea.

Morphology.—Oval diplococci with opposed surfaces flattened or even concave. The diameter of the coccus is about 0·8–1μ. Morphologically the gonococcus is identical with the meningococcus. In inflammatory exudates the intracellular position of the organism is characteristic, and pus cells often appear to be almost filled with ingested diplococci. In culture, involution forms are frequent.

Staining.—Gram-negative.

Culture.—Aerobe; temperature range -30°-39° C., optimum—36°-37° C.; requires blood or serum for growth—*e.g.* agar containing 10 per cent. blood (heated at 55° C.), serum-agar prepared from fresh sterile serum (*vide* p. 129), or agar containing 10 per cent. hydrocele fluid. Various special media have been recommended, but the above-mentioned serve satisfactorily in the routine cultivation of the organism. The agar should be carefully standardised to *p*H 7·5.

Care should be taken to ensure that the medium used is sufficiently moist and is kept moist during incubation. For this purpose plate cultures may be incubated inside a closed jar containing a pad of cotton-wool moistened with water.

Many strains grow better in an atmosphere containing carbon dioxide (*e.g.* 5 per cent.) than in ordinary air (*vide* p. 178).

Colonies are semi-transparent disks about the size of a pin head, tending to remain discrete, circular in outline at first, but later showing a "scalloped" or crenated margin, a raised more opaque centre, and sometimes radial and concentric markings. Papillae may be noted after some days' growth.

In primary cultures, colonies may be slow in developing and growth may not appear for three or four days.

Two different types of colonies have been described: a large, thin irregular colony on which papillae develop, and a small round colony which is more raised and without papillae.

Biochemical Reactions.—Can be tested for as in the case of the meningococcus, the medium described on p. 131 being used; ferments glucose, but not maltose (*vide* Table *supra*).

Viability.—The thermal death-point is about 55° C.. This organism is very susceptible to drying, and when desiccated *under ordinary conditions* dies within two hours. It is a strict parasite and tends to die rapidly (*e.g.* in a few hours) when discharged from the body, especially if subjected to cooling and drying. It has been found, however, that under certain conditions—*e.g.* in pus on linen or other fabric—the gonococcus may remain viable for periods up to three days. When first cultivated, cultures have a feeble viability, and subcultures should be made every three or four days to maintain the strain. When accustomed to artificial growth, cultures survive longer if kept at 37° C., and in a moist condition—*e.g.* two to three weeks. Cultures die at room temperature in two days.

Occurrence.—In the *male* the organism infects the mucosa of the urethra and produces a suppurative inflammation with purulent discharge. The cocci are present in large numbers in the discharge at an early stage, but later diminish, and are then associated with secondary infecting organisms—*e.g.* pyogenic cocci, *B. coli*, diphtheroid bacilli. They may invade the prostate, vesiculae seminales, epididymis, bladder mucosa, and peri-urethral tissue (producing a peri-urethral abscess).

In the *female* the urethra and cervix uteri are infected, but rarely the vaginal mucosa. Bartholin's glands, the endometrium, and the Fallopian tubes may be invaded, and even the peritoneal cavity.

Purulent conjunctivitis may occur as a complication. Blood invasion may result from primary gonorrhoeal infections, and arthritis is a common complication.

While the gonococcus has on occasion been cultivated from the joint fluid in arthritis, the possibility of gonococcal arthritis being a manifestation of allergy must also be considered (*cf.* acute rheumatism in relation to streptococcal infection, *vide* p. 343). Ulcerative endocarditis has been noted as an occasional sequela.

In female infants and children the gonococcus may produce a persistent vulvo-vaginitis.

In new-born infants gonorrhoeal ophthalmia may result from direct infection at birth.

Chemotherapy.—The sulphonamide compounds, *e.g.* sulphapyridine, have proved most effective chemotherapeutic agents in gonococcal infections. Penicillin therapy has been applied with conspicuous success, *e.g.* in cases due to sulphonamide-resistant strains of the gonococcus.

DIAGNOSIS

Films are made from the discharge.—In the *male* : from the urethral discharge ; the meatus should be cleansed with sterile gauze soaked in saline solution, and specimens are taken either with a platinum loop, or directly on slides. In the *female* : from the urethra and cervix uteri, with a platinum loop and with the aid of a vaginal speculum.

The films are stained by (*a*) methylene blue, and (*b*) Gram's method (with neutral red, or Sandiford's stain—*vide* p. 201—as the counter-stain), and in the acute stage, both in the male and female, the occurrence of the *characteristic Gram-negative intracellular organisms* is, for clinical purposes, diagnostic.

In *Chronic infections*, particularly in the female, the cocci may be relatively scanty in films, and difficult to identify accurately among the secondary infecting organisms. In the male the " morning drop " of secretion from the urethra should be examined, or films from the centrifuged urinary deposit or the discharge after prostate massage. In the female the secretion from the cervix uteri should be examined.

The diagnosis may be confirmed by cultivation, but where there is a mixed infection this may be technically difficult. Inoculation with material to be cultivated should, if possible, *be made directly from the patient on a suitable medium* (*vide supra*), and the culture should be incubated at once. If the material is kept at room temperature for some time before inoculation and incubation, or if it is allowed to dry, the organisms, being strict parasites, may die and fail to grow on the culture medium.

The Oxidase Reaction in the Detection of Colonies of the Gonococcus.—Cultures are made on plates of heated-blood-agar and after two days' incubation, 1 per cent. tetramethyl-*p*-phenylenediamine solution is poured on to the plate so as to cover the surface, and then decanted. The colonies of the gonococcus develop a purple colour (oxidase reaction). If subcultures are required from the colonies these should be made immediately; after ten minutes it may not be possible to subcultivate them. This method is specially useful in dealing with heavily contaminated material containing only scanty gonococci.

Serum Diagnosis.—The complement-fixation test is applicable for diagnosis and may be of considerable value to the clinician. Various types of antigen prepared from cultures of the gonococcus have been used. The antigen recommended is that devised by Price, who claims that in the first week of the disease 27 per cent. of positive results are obtained, rising to 46, 70, 89 and 100 per cent. after two, three, four and five weeks respectively.

The method of making the antigen and the technique of the test are described on p. 283.

The actual test is carried out by the technique used for the Wassermann reaction.

ORGANISMS OF GENUS VEILLONELLA

These are of some interest in view of their occurrence as commensals in natural cavities of man and animals, particularly the mouth and alimentary tract. They have not been definitely proved pathogenic though sometimes isolated from the appendix, pyorrhoea, pulmonary lesions, etc., and regarded as potentially pyogenic.

They are minute Gram-negative cocci about $0{\cdot}3\mu$ in diameter and occurring in masses. In cultural characters they are anaerobic and grow best at 37° C.. The type species is *V. parvula* whose distinctive characters are: the formation of hydrogen, carbon dioxide, hydrogen sulphide and indole from polypeptides, the fermentation of glucose and certain other sugars; haemolytic action and the reduction of nitrate to nitrite.

CHAPTER XII

DIPHTHERIA BACILLUS AND BIOLOGICALLY ALLIED ORGANISMS; BACILLUS PSEUDO-TUBERCULOSIS OVIS AND BACILLUS OF EQUINE ULCERATIVE LYMPHANGITIS; BACILLUS PYOGENES; BACTERIUM MONOCYTOGENES

BACILLUS DIPHTHERIAE (*Corynebacterium diphtheriae*)

THE causative organism of diphtheria.

Morphology and Staining.—Slender rod-shaped organism, straight or slightly curved; the average size is 3μ by 0·3μ, but longer and shorter forms may be noted; the ends are often expanded; it is non-motile and non-sporing. In culture, involution forms may be observed which are pear-shaped, club-shaped, or even globular. The bacillus is Gram-positive, though more readily decolorised than many other Gram-positive organisms; stained with methylene blue it shows a "beaded" or "barred" appearance. "Barred" staining is characteristic of the *intermedius* type of diphtheria bacillus (*vide infra*). By Neisser's method (*vide* p. 206) metachromatic granules are characteristic, staining blue-black in contrast with the light-brown coloration of the rest of the organism; the granules are often polar in situation. If over-decolorised in the Gram method the granules tend to retain the violet stain, while the rest of the organism is decolorised. These characteristic staining reactions depend on environment; thus, in culture metachromatic granules are most pronounced when the bacillus is growing on a serum medium such as Löffler's. Appearances also vary among strains; some show very marked "barred" staining; certain strains exhibit very short forms with poorly developed granules.

Culture.—Aerobe; temperature range—20°-40° C., optimum—37° C.; grows on ordinary nutrient media, but best on serum media.

Colonies on Löffler's serum—at first small, circular, white, opaque disks with regular borders; later the centre becomes thicker and the borders crenated; they may reach in diameter 3–4 mm. after several days' growth; sometimes the growth on serum shows a distinct yellow tint.

Does not liquefy gelatin.

In broth some strains grow in small white masses, which sediment in the tube and also adhere to the side; a surface film of growth may also develop. Other strains produce a uniform growth in broth.

On a medium containing potassium tellurite the diphtheria bacillus reduces the tellurite and yields greyish or black colonies. Potassium tellurite is also selective in certain concentrations for this organism, and the allied diphtheroid bacilli (*vide* p. 141).

On certain selective culture media the colonies have a distinctive appearance. On Clauberg's medium (p. 144), they have a blue coloration, while on Neill's medium (p. 146), the colonies are greyish black and granular with a characteristic "punched out" appearance. (See also **Appendix**.)

Three types of colony on McLeod's medium (p. 143) have been recognised and considered as characteristic of different biological types, designated *B. diphtheriae gravis, mitis* and *intermedius.* The designations *gravis* and *mitis* have been applied in virtue of the association of these types with severe and mild forms respectively of the disease. The *gravis* type produces relatively large greyish-black, flat, lustreless colonies exhibiting often a "daisy-head" formation. Growth in broth is granular. The *mitis* type yields a convex, smooth, translucent colony, and growth in broth presents a uniform turbidity. The *intermedius* type is represented by relatively small, black, lustreless colonies with domed centre and flat, irregular margin.

Biochemical Reactions.—Ferments with acid production, glucose, galactose, maltose and dextrin, but not lactose, saccharose or mannitol.

Gravis strains ferment starch and glycogen, and these reactions have been emphasised as features of this type. The *mitis* and *intermedius* types have no action on these carbohydrates.

These reactions can be elicited by using Hiss's serum medium (*vide* p. 128) with 0·1 per cent. peptone added. Some workers use phenol-red as the indicator, the initial pH being 7·6.

In addition to the biological types originally described as *gravis*, *mitis* and *intermedius*, certain additional types have been described in cases of diphtheria and carriers. Two of these, however, are avirulent forms.

The following table illustrates the classification of these types :—

Type	*Colony form*	*Starch fermentation*	*Virulence to guinea-pig*	*Haemolysis*	
				Ox	*Rabbit*
				Blood	
I (*mitis*) .	mitis	—	+	+	+
II (*intermedius*) .	intermedius	—	+	—	—
III (*gravis*) .	gravis	+	+	—	+
IV . .	,,	—	+	—	—
V . .	,,	+	—	—	—
VI . .	,,	—	—	—	—
VII . .	mitis	+	+	.	.

(H. A. Wright and M. H. Christison ; J. Wright)

Strains are also occasionally met with which cannot be exactly classified.

It should be noted that the stability of the supposed biological types of the diphtheria bacillus has been questioned ; thus it is stated that strains undergo variation in colony form and starch fermentation.

Serological classification of starch fermenting (gravis) stains.—By agglutination reactions with antisera these stains have been classified into five sub-types of which Type I (Robinson and Peeney) is commonest at the present time in Great Britain.

Haemolysis.—Strains of the *mitis* type are generally haemolytic when growing in a medium containing ox or rabbit blood; the *intermedius* strains are invariably non-haemolytic; strains of the *gravis* type usually lyse rabbit but not ox blood. (H. A. Wright.)

Viability.—In culture, diphtheria bacilli may remain alive for two or more months at room temperature. In the moist condition they are comparatively easily killed by heat (in ten minutes at 60° C.), but when dry survive for much longer periods. The bacilli in dried membrane kept at room temperature and in the dark have been found to be alive and virulent after several months.

Occurrence.—The bacilli are present in large numbers in the " false membrane " and in the throat secretions. They do not invade the lymphatics to any extent, and there is no general blood infection. In nasal diphtheria the organisms can be detected in the nasal discharge. Infection of wounds, the conjunctiva, vulva and vagina may occasionally occur. A diphtheritic paronychia is sometimes met with.

It is generally agreed now that the *gravis* type tends to be associated with a more severe and toxic form of diphtheria than the *mitis*, but the *intermedius* may practically equal the *gravis* type in pathogenicity to the human subject. The relative prevalence of these types varies in different areas, and varies also at different times.

Diphtheria Toxin.—The diphtheria bacillus produces a powerful exotoxin with specialised toxic properties. While the bacillus remains localised at the site of infection, the diffusible toxin is absorbed into the blood stream and leads to the various systemic disturbances of diphtheria and to such sequelae as post-diphtheritic paralysis.

When diphtheria bacilli are grown in suitable fluid media abundant toxin is produced. The strain used is, however, of considerable importance, for in artificial culture the organism may not adapt itself so readily to toxin production as it does in the human body. A single strain (Park-Williams No. 8) is almost universally used for toxin production on a large scale. The bacilli are removed from the culture by filtration or other means, and the bacteria-free liquid, which contains the exotoxin mixed with the culture medium and other products of bacterial growth, is referred to as "toxin."

Certain cultural conditions are required for maximal production of diphtheria toxin. It was thought at one time that peptone-like substances produced by enzymic digestion of meat were essential for toxin production, but the work of Müller on the nutritional requirements of *B. diphtheriae* has led to great advances in our knowledge. It is now possible to produce very potent toxin on a medium containing known amino-acids, inorganic salts, maltose, and in addition, certain substances regarded as growth factors (pimelic acid, nicotinic acid and β-alanine). The toxin produced on this type of synthetic medium has been isolated in what would appear to be a pure state; it has the properties of a labile protein with a molecular weight of 70,000 and a lethal dose of 0·0001 mgm. for a 250 grams guinea-pig [10,000,000 lethal doses per gram] (Pappenheimer).

For large scale production, 5-litre capacity bottles (D.W.Q.) containing 500–700 c.c. of medium (Hartley's broth—p. 101) or preferably that of Pope and Smith[1] are inoculated with the Park-Williams 8 strain and incubated at 33°–35° C. for 10 days. The bottles are placed in a horizontal position to secure maximum surface for growth and to allow free access to oxygen which is important for toxin production. The cultures are removed from the incubator after 10 days and the organisms killed by the addition of toluol. Filtration through paper and then through an earthenware or Seitz filter yields a bacteria-free filtrate which constitutes the crude toxin.

The toxic substance so produced is somewhat unstable and its potency diminishes on exposure to air and light. In sealed tubes and in the dark it may remain unaltered for several weeks. The loss of toxic action is due to the spontaneous conversion of toxin into "toxoid" which has no pathogenic effect on animals but still retains the power of combining with antitoxin and of stimulating immunity.

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[1] See Pope, C. G., and Linggood, F. V., *Brit. J. Exper. Path.*, 1939, **20**, 303.

This process of conversion of toxin into toxoid can be carried out by adding 0·3 per cent. of formalin and incubating the toxin for two to three weeks at 37° C.. The change from the toxic into the non-toxic state is determined by injecting the material into guinea-pigs; when 5 c.c. injected subcutaneously or intraperitoneally produce no symptoms, the change is regarded as complete. Toxoid produced through the action of formalin in this way is a valuable immunising agent.

Diphtheria toxin is a powerful poison, particularly for guinea-pigs in which 0·00025 c.c. of a culture-filtrate may be fatal within five days. At the site of inoculation there is a greyish necrotic focus surrounded by an area of congestion, while the subcutaneous tissue shows marked inflammatory oedema. The neighbouring lymph glands are swollen and congested, the suprarenals are enlarged, very congested and may show haemorrhages; the lungs are usually congested and there is effusion into the pleural cavities; the kidneys and liver show degenerative changes. The amount of toxin formed varies with the strain of organism used and the composition of the medium, and each batch of toxin has to be tested by animal experiment.

The same pathogenic effects as those described above are produced by the subcutaneous injection of a living culture of virulent diphtheria bacilli. The organisms remain more or less localised at the site of inoculation, while their toxin is absorbed into the circulation.

Virulence.—When bacilli morphologically resembling *B. diphtheriae* are found in the throat or nose unassociated with active disease (*i.e.* in carriers) it is important to determine whether they are virulent or not. The virulence test is usually done by the intradermal injection of a pure culture into guinea-pigs as follows.

The primary cultures from the nose or throat are plated out and a single colony subcultured to obtain a pure growth. Tellurite media such as Hoyle's (see **Appendix**), McLeod's (p. 143) or Allison and Ayling's (p. 142) are recommended for the easy isolation of the diphtheria bacillus. The fermentative powers of the selected pure growth are tested, and if the organism ferments glucose, but not saccharose, a suspension from a culture on Löffler's serum or serum-agar is made in broth of such a strength that the fluid is distinctly opalescent (about 500,000,000 bacilli per c.c.). Two white guinea-pigs of about 400 grams weight are selected, and the hair removed from the flanks as described on p. 235. The day before the test is carried out, one of the animals—the control guinea-pig—is injected intraperitoneally with 1000 units of diphtheria antitoxin (*vide infra*). For the actual test each guinea-pig is injected intradermally with 0·2 c.c. of the suspension of organisms, a 1 c.c. syringe and fine-bore needle (26 gauge, $\frac{3}{8}$ in. long) being used. Several different cultures may be tested on each guinea-pig, and the injections should be about one inch apart. A careful note of the position of the injection of each different organism should be made. It is not advisable to carry out more than ten simultaneous tests on an animal. Four hours afterwards the test guinea-pig (*i.e.* the one that did not previously receive antitoxin) is now injected with 100 units of antitoxin. Alternatively the test guinea-pig may be injected immediately with $\frac{1}{50}$th unit of antitoxin per gram of body weight. The guinea-pigs are examined twenty-four, forty-eight and seventy-two hours after inoculation.

Virulent diphtheria bacilli produce in the test animal a well-defined red area about 15 mm. in diameter. After the third or fourth day the colour fades, leaving a necrotic patch with a scab surrounded by growing hair. The control guinea-pig shows no such reaction, the puncture wound caused by the needle being generally the only evidence of injection. If the organism is non-virulent there is no reaction in either the test or control animal. A reaction in both animals shows that the organism is not the diphtheria bacillus, because the products of growth are not neutralised by diphtheria antitoxin, as indicated by the reaction of the control animal.

Rabbits are also suitable for virulence tests, and some workers have used these animals for the purpose.

Diphtheria Antitoxin.—By immunising horses with toxoid and then with toxin, an antitoxin is produced from the tissues and appears in the blood ; the serum of such animals constitutes " diphtheria antitoxin." For regulating the dosage of this antitoxin, an arbitrary " immunity unit " has been adopted and the number of such units in a given volume of serum denotes its antitoxic value. The unit was *originally* defined as that amount of antitoxin (or antitoxic serum) which just neutralises 100 M.L.D. of a certain toxin, the M.L.D. (minimum lethal dose) being the minimum amount which kills a guinea-pig of 250 grams weight in four days. It has not been feasible to preserve a standard toxin for testing antitoxin, but by means of a preserved standard antitoxin any toxin preparation can be standardised by neutralisation tests in guinea-pigs, and in turn the value of a new antitoxin can be estimated. The usual method for the purpose is to ascertain first the " L+ " dose of the toxin ; this is the quantity which when mixed with one unit of standard antitoxin is just sufficient to kill a 250 grams guinea-pig in four days. Varying dilutions of the new antitoxin are then mixed with the L+ dose and injected into guinea-pigs. Thus, the neutralising power of the new antitoxin can be compared quantitatively with the standard and the number of units in a given volume stated. Antitoxin may also be titrated by the intracutaneous injection of mixtures of toxin and antitoxin in the guinea-pig or rabbit, and, as a preliminary to the *in vivo* tests, by the flocculation reaction in mixtures of toxin and antitoxin; flocculation occurs most quickly at the neutrality point.

The antitoxic property of serum is contained in the globulin fraction, and by precipitation in half-saturated ammonium sulphate the serum can be concentrated and much of the protein material which gives rise to serum sickness is removed. This was the method originally used, but "refined" antitoxins are now available, prepared by treatment of the serum with proteolytic enzymes, *e.g.* pepsin, which digests albumin, leaving the antitoxin-globulin unaltered. The volume to be injected is thus reduced, and the same number of units represents less than half the amount of protein present in the concentrated sera previously used. The incidence of serum sickness has also been much lessened. The antitoxin-bearing molecule is smaller and this apparently yields a greater therapeutic efficiency.

The Regulations under the Therapeutic Substances Act define the strength, quality and testing of diphtheria antitoxin, and these regulations must be observed by manufacturers supplying serum for therapeutic use.

It has been observed that diphtheria due to the *gravis* type of bacillus (*vide supra*) may be of a hypertoxic type and refractory to antitoxin treatment. All strains of the diphtheria bacillus, however, produce the same toxin, but it has been claimed that two distinct substances enter into its constitution and that the toxins from different strains may vary in the relative amounts of these constituents.[1]

Schick Reaction.—When a minute quantity of diphtheria toxin is injected intradermally, a local reaction follows in persons with less than a certain content of antitoxin in the blood. The average amount of antitoxin required to ensure neutralisation of the test dose of toxin and to protect against diphtheria under ordinary conditions is about 1/200 of a unit per c.c. of blood. It should be noted, however, that there are wide variations in antitoxin content among non-reactors. For the test an old toxin preparation is chosen in which some of the toxin has changed to toxoid, and the selected dose is that amount which is just neutralised by 1/1000 unit of antitoxin. The toxin preparation is diluted with a special buffer solution[1] so that 0·2 c.c. contains the test dose. This is injected intradermally in the left forearm and as a control an equal amount of a similar dilution of the same toxin previously heated at 85° C. for ten minutes is injected intradermally in the right forearm. A positive Schick reaction consists in an area of redness and swelling appearing after twenty-four to forty-eight hours, reaching its maximum about the fourth day, when it measures 1–5 cm. in diameter. It persists for seven to fifteen days, and on fading shows superficial scaling and a persistent brownish pigmentation. The absence of reaction on either arm (*i.e.* "a negative Schick reaction") indicates that the toxin has been neutralised, sufficient antitoxin being present in the blood of the individual. A "pseudo-reaction" may occur, *i.e.* an area of redness appearing early (within six to twelve hours) which is less intense and usually disappears in one to three days. If this appears in a Schick-negative person both forearms show similar reactions; in a positive reactor the unheated toxin produces a reaction which is more pronounced and more persistent than that due to the heated material. A convenient time to examine results is after seven days, when true reactions are still visible and most pseudo-reactions have faded. It is usually possible to determine without difficulty whether the result is positive or negative.

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[1] See O'Meara, R. A. Q., *J. Path. Bact.*, 1940, **51**, 317.

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[1] A mixture of 57 grams crystal borax, 84 grams boric acid, 99 grams sodium chloride is made; 1·5 grams of this mixture are dissolved in 100 c.c. of distilled water. In this country a small amount of human serum (containing no detectable antitoxin) is also used as a stabiliser.

Pseudo-reactions are more common in older children and adults, because they have apparently been sensitised by exposure to diphtheria bacilli. They are usually immune to diphtheria, as such exposures have also given rise to the production of specific antitoxin.

Children between nine months and eight years of age are generally non-immune and rarely suffer from reactions after prophylactic immunisation. Up to the age of eight years, therefore, the preliminary Schick test may be omitted before immunisation. In older children it is recommended that a preliminary Schick test should be carried out.

Under the Therapeutic Substances Regulations the reagents of the Schick test are designated "Schick Toxin" and "Schick Control," and the methods of manufacture must be in accordance with these regulations.

Diluted toxin prepared with the proper buffer solution and kept in the cold will remain fully active for many months.

Diphtheria Prophylactics.—Individuals having little or no antitoxin in the blood stream, as indicated by a positive Schick reaction, may be actively immunised by the injection of one of the following preparations:

(1) Formol-toxoid (F.T.): diphtheria toxin modified in toxicity by formalin (*vide* p. 379). The recommended dosage is as follows:—For adults three injections of 0·1 c.c., 0·2 c.c., and 0·3 c.c.; for children 0·2 c.c., 0·4 c.c., and 0·6 c.c. The interval between doses is three weeks, and injections are made intramuscularly. As the reactions are likely to be severe except in young children, the sensitivity to toxoid may be tested by the intradermal injection of 0·2 c.c. of a 1 in 100 dilution of toxoid. This test (sometimes designated the Moloney test) should be negative if the full dose is to be given.

(2) Toxoid-antitoxin mixture (T.A.M.) in which the toxoid is partially "neutralised" by antitoxin. Three injections of 1 c.c. are made intramuscularly into the upper arm, at intervals of four weeks. T.A.M. is not such a powerful antigen as F.T. but the reactions produced are less.

(3) Toxoid-antitoxin floccules (T.A.F.): a suspension of the precipitate of floccules formed when toxoid and antitoxin are mixed in appropriate "neutralising" amounts. Its tendency to cause reactions is slight, and a high degree of immunity follows the injection of three doses, each of 1 c.c., given at intervals of four weeks.

(4) Alum-precipitated toxoid (A.P.T.): a suspension of the washed precipitate produced by the addition of a small amount of alum to toxoid. The precipitate is relatively insoluble and the toxoid is gradually liberated from the site of injection. Reactions are negligible in children under eight years, but in older children and adults they may be somewhat more severe than with T.A.M. or T.A.F. Even a single injection of 0·5 c.c. gives fairly high immunity, but it is preferable to inject two doses, each of 0·5 c.c. in children or 0·1 c.c. followed by 0·5 c.c. in adults, at an interval of four weeks. Adolescents or adults who show a local reaction after 0·1 c.c. may receive three injections of 1 c.c. of T.A.F. at intervals of four weeks to complete their immunisation.

The testing of all diphtheria prophylactics must be done in accordance with the Therapeutic Substances Regulations.

Whatever prophylactic is used, it is important that a subsequent Schick test should be carried out eight to twelve weeks after the last injection to confirm the production of a satisfactory immunity.

For further details of the preparation of toxin, toxoid and antitoxin, one of the larger works should be consulted.

DIAGNOSIS

A specimen of the throat secretion should be obtained. No antiseptics (*e.g.* in form of gargles, etc.) must have been applied within twelve hours. A sterile throat swab should be rubbed over the affected area, or, where there is no definitely localised lesion, over the mucous membrane of the pharynx and tonsils.

Two procedures may now be carried out :—

Löffler's medium is inoculated as in the original method of diphtheria diagnosis, and direct plating is made on one of the selective tellurite media, *e.g.* Hoyle's modification of Neill's medium (see **Appendix**).

1. A tube of Löffler's medium is inoculated by smearing the infected swab over the whole surface of the medium, moistening the swab in the condensation water at the foot of the tube. The tube is incubated for eighteen to twenty-four hours at 37° C..

If an earlier result is urgently required, the culture may be examined after six to twelve hours : if this should be negative, however, the examination must be repeated after eighteen to twenty-four hours.

The resulting growth is mixed by emulsifying it with a wire loop in the condensation fluid, and from this, films are made and stained by Neisser's method or Albert's modification (*vide* p. 206). Albert's method can be specially recommended.

Films may also be made directly from the swab and stained by the above methods, but only in a small proportion of cases can positive results be obtained in this way, and cultures should always be made as a routine procedure, irrespective of direct examination.

In the case of suspected throat diphtheria, the appearance in cultures of bacilli showing the characteristic morphology and staining reactions (especially the metachromatic granules by Neisser's or Albert's stain) may be regarded as significant in confirming the clinical diagnosis.

It should be noted that in some cases other organisms may overgrow the diphtheria bacillus in culture and lead to an apparently negative result. Moreover, the *gravis* type is often difficult to recognise in early growths, the bacilli being short and thick with absence of metachromatic granules. In mild cases and carriers the bacilli may be scanty and easily missed.

In the case of supposed nasal diphtheria, diphtheria carriers, diphtheria affecting mucous surfaces other than the throat, and wound-diphtheria, the microscopic examination of cultures is not conclusive. The suspected organism must be isolated in pure culture and its virulence determined as described on p. 379.

2. The above method cannot be relied on in all cases, and it is advisable to cultivate the swab at the same time on a tellurite medium, incubating for twenty-four to forty-eight hours and making a diagnosis by recognition of the characteristic colonies, including the identification of the different colony-types, *gravis*, *mitis* and *indermedius*. (On tellurite media the typical metachromatic granules may not be demonstrable.) The two methods used together serve to check one another. It should be remembered that while tellurite inhibits many other organisms, diphtheroid organisms may grow on it as well as the diphtheria bacillus, and must be carefully differentiated from the latter. Hoyle's tellurite medium is specially recommended. Growths are only just visible after twelve hours, but by using a plate-culture microscope colonies of *B. diphtheriae* can often be recognised by certain characters (see **Appendix**); at this stage, however, the value of the plate is mainly in attracting attention to the presence of diphtheria bacilli missed on the Löffler's medium. After eighteen to twenty-four hours the growth is more abundant, the characters of the colonies are more distinct and by the combined use of the two methods a high degree of diagnostic accuracy is attained. When the bacilli are scanty or when nasal or aural swabs are examined, thirty-six to forty-eight hours may be required for the recognition of *B. diphtheriae* colonies, and if there is any doubt at twenty-four hours, further incubation should be allowed before reporting. The tellurite plate also facilitates the isolation of pure cultures and when there is difficulty in identifying the diphtheria bacillus by colony characters, and where this organism occurs in a carrier, a pure culture must be obtained and tested for its biochemical reactions and virulence. (If, however, the organismspresent all the characters of the *gravis* type, virulence is generally assumed without resorting to an animal test.)

It must be emphasised here that the responsibility for the diagnosis of diphtheria rests entirely with the clinician. The bacteriologist can merely state by certain routine methods whether organisms morphologically resembling the diphtheria bacillus are present in cultures from the specimen submitted to him or whether the growths on a tellurite medium are typical of this organism. Failure to find such organisms does not necessarily exclude diphtheria, nor does their presence prove the disease to be diphtheria.[1] *If the clinician considers a case to be diphtheria it is his duty to administer antitoxin at once*, and continue to do so even if a negative laboratory report is received. The mortality from the disease increases with the delay in administering antitoxin, and where there is reasonable suspicion that the case may be diphtheria, antitoxin must immediately be used without waiting for a bacteriological report. It is also emphasised that a reliable laboratory report, particularly in cases where there is doubt clinically, cannot be made under eighteen to twenty-four hours, and at that stage the bacteriologist can report on morphological or cultural appearances only. To prove conclusively the identity and virulence of the organism necessitates tests extending over several days. In order that the bacteriological report should be as helpful as possible, the utmost care must be taken that a suitable specimen is submitted in accordance with the directions given above and precise details as to the nature and source of the material should be furnished.

Allowance being made for the possible limitations of the routine diagnostic methods described, the results of such examination have undoubtedly proved of the greatest value as an aid to, and confirmation of, the clinical diagnosis.

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[1] See *A System of Bacteriology*, Medical Research Council, London, 1930, v, 100.

BACTERIA BIOLOGICALLY ALLIED TO THE DIPHTHERIA BACILLUS (DIPHTHEROID BACILLI)

BACILLUS OF HOFMANN (*Corynebacterium hofmanni*)

A throat commensal.

Morphology and Staining.—Compared with *B. diphtheriae* it is shorter (about 2μ) and may present a somewhat oval appearance; stained with Löffler's methylene blue, an unstained bar in the middle of the organism is a frequent character and renders it not unlike a diplococcus. It is strongly Gram-positive; usually no metachromatic granules are detected by Neisser's method.

Culture.—Grows aerobically on ordinary media; growths are more abundant than those of *B. diphtheriae*, and the colonies are larger and more opaque. For appearances on one of the tellurite media see p. 143.

Biochemical Reactions.—*Vide* Table p. 390.

It is non-pathogenic to laboratory animals.

BACILLUS XEROSIS (*Corynebacterium xerosis*)

A commensal in the conjunctival sac. Closely resembles *B. diphtheriae*, and may show metachromatic granules.

Can be differentiated from *B. diphtheriae* by its production of acid in saccharose (*vide* Table *infra*) and by its non-pathogenicity to laboratory animals.

ACNE BACILLUS (*Corynebacterium acnes*)

An organism associated with acne and regarded as the aetiological agent. It is Gram-positive, rod-shaped, and measures about $1{\cdot}5\mu$ by $0{\cdot}5\mu$. It is markedly pleomorphic, and frequently shows a beaded appearance, resembling a diphtheroid bacillus.

Culture.—In primary culture grows under anaerobic conditions or as a micro-aerophile if a fermentable carbohydrate such as glucose is present, or aerobically if the medium contains serum or blood and is definitely acid (pH 6·2–6·8).

OTHER DIPHTHEROID BACILLI

Certain of these present a close similarity to *B. diphtheriae*, and may exhibit the characteristic granules by Neisser's staining method (*vide* p. 206) though differing in fermentative reactions, *e.g.* fermenting saccharose. They are mostly non-pathogenic, and have been isolated from the secretions of the nose and naso-pharynx, the ear, conjunctival sac, the skin, lymph glands (apart from disease) and other tissues, pus, wounds, etc.. Compared with the true diphtheria bacillus they are of low virulence to laboratory animals.

Barratt has described diphtheroid bacilli in the nasopharynx which tend to resemble the Preisz-Nocard bacillus (*vide infra*) ; these organisms liquefy gelatin ; they are virulent to guinea-pigs and rats, but diphtheria antitoxin has no protective action against them.

BIOCHEMICAL REACTIONS OF B. DIPHTHERIAE AND CERTAIN ALLIED TYPES

	Glucose	*Saccharose*	*Dextrin*
B. diphtheriae . .	⊥	–	⊥
B. of Hofmann . .	–	–	–
B. xerosis . . .	⊥	⊥	–

(⊥ = acid production)

BACILLUS PSEUDO-TUBERCULOSIS OVIS (PREISZ-NOCARD BACILLUS)

(*Corynebacterium ovis*)

The causative organism of caseous lymphadenitis and pseudo-tuberculosis in sheep. A similar organism is associated also with ulcerative lymphangitis of horses (*vide infra*).

This organism is allied to *B. diphtheriae* in its biological characters.

Morphology.—Non-motile, slender rod-shaped organism 1–3μ in length by 0·4μ in breadth. When stained, it shows beading or a barred appearance like other diphtheroid bacilli, and is Gram-positive. By Neisser's method metachromatic granules can be demonstrated. Club-shaped forms may be noted.

Culture.—Growth occurs at 37° C. under both aerobic and anaerobic conditions on ordinary nutrient media :

On agar, growth is at first scanty ; the colonies are small, thin, dry and greyish-white in colour, folded and granular and often show concentric rings.

In gelatin liquefaction occurs.

On Löffler's serum the colonies are similar to those on agar but exhibit a yellow colour.

In broth a granular growth occurs with sometimes a surface pellicle.

Glucose, maltose and dextrin are fermented, but not saccharose, lactose, mannitol or glycerol. A haemolysin is produced in culture medium.

Occurrence.—The associated disease in sheep is chronic and characterised by involvement of lymphatic glands, which are enlarged and caseous. Caseous nodules are seen also in the internal organs—*e.g.* lungs, spleen, liver and kidneys. The organism can be demonstrated in films or sections prepared from the various lesions.

Animal Inoculation.—Laboratory animals—*e.g.* guinea-pig and rat—are susceptible to experimental infection with cultures. Intravenous injection in the guinea-pig produces a lethal effect within about ten days and at autopsy caseous areas are noted in internal organs—*e.g.* lungs and liver. Intraperitoneal injection in a male animal leads to involvement of the tunica vaginalis as in the case of experimental glanders (*vide* p. 412). Subcutaneous injection is followed by lymphatic gland involvement, the glands showing the characteristic caseation. In rats inoculation produces a fatal septicaemia. Sheep and goats are also susceptible to experimental inoculation.

This organism produces an exotoxin resembling to some extent that of the diphtheria bacillus, but not neutralisable by diphtheria antitoxin. Guinea-pigs are highly susceptible to this toxin and show at the site of subcutaneous inoculation an inflammatory lesion with oedema and haemorrhage, while the internal organs are congested and often contain small haemorrhages ; there is, however, no change in the suprarenals and no pleural effusion (*cf.* diphtheria).

Diagnosis.—Films are prepared from the lesions and stained by Gram's method, methylene blue, and by Ziehl-Neelsen's method (to exclude acid-fast bacilli). Cultures are made, and pure growths from single colonies are investigated as regards cultural characters and experimental pathogenesis.

Similar organisms are found in equine ulcerative lymphangitis (pseudo-farcy), and in pseudo-tuberculosis (caseous lymphadenitis) of bovines ("*C. bovis*"). These organisms form a group of closely related types. They have frequently been classified as one species—the "*Preisz-Nocard Bacillus.*" A similar organism (*B. pseudo-tuberculosis murium*) produces disease in mice.

BACILLUS PYOGENES (*Corynebacterium pyogenes*)

An organism associated with suppurative lesions in pigs, cattle and certain other animals. It may occur in mastitis of cattle and sheep.

Morphology and Staining Reactions.—Non-motile, rod-shaped organism not usually exceeding 2μ in length. Shows great pleomorphism. Gram-positive in young cultures. Stained with methylene blue, diphtheroid forms may be seen with deeply-stained bands or granules, but metachromatic granules are not usually observed in preparations stained by Neisser's method.

Culture.—Aerobe and facultative anaerobe but some strains grow better under anaerobic conditions. Optimum temperature about 37° C.. Generally requires media containing blood or serum. The colonies on serum media are at first minute, but after several days' growth may attain a size of 2–3 mm. in diameter. They present no specially characteristic appearances. Growth on coagulated serum produces small pits of liquefaction. Gelatin is also liquefied. This organism is haemolytic when growing on blood-agar and a filterable haemolytic toxin can be demonstrated in suitable culture medium.[1] In milk, acid and clot result in three days and after a time the clot is digested. Glucose, lactose and, in some cases, saccharose are fermented. Mannitol is not fermented.

A variant type which lacks proteolytic action has been described.

Pathogenesis.—In the natural infection in swine, suppurative lesions may occur in various parts of the body, liver abscesses and arthritis being specially frequent. In cattle, the organism has been found associated with a variety of suppurative lesions —*e.g.* abscesses, pyaemia, pyelitis, mastitis, etc..

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[1] See Lovell, R., *J. Path. Bact.*, 1941, **52**, 295.

Rabbits can be infected experimentally ; intravenous injection of cultures produces a pyaemic condition with bone and joint lesions. Guinea-pigs are less susceptible.

Corynebacterium renale.—This organism has been described in pyelonephritis of cattle. It resembles other organisms of the diphtheroid group in general characters. It digests milk casein, but has no action on gelatin or coagulated serum. Glucose is fermented ; some strains also ferment laevulose and mannose.

Corynebacterium equi has been reported as the causative organism of pneumonia in colts. It differs from other members of the diphtheroid group in its profuse viscid growth and the production of a red pigment. Carbohydrates are not fermented.

BACTERIUM MONOCYTOGENES (*Listerella monocytogenes*)

This organism owes its specific name to the fact that infection by it in laboratory animals, *e.g.* rabbits and guinea-pigs, produces a monocytosis in the blood. It was originally isolated from these animals, but similar bacteria have been found in gerbilles, sheep and certain other animals, and in meningitis of the human subject.[1] It has been reported recently in a human case presenting the features of infectious mononucleosis. This organism has been found in foetuses from cases of abortion in sheep.

It occurs as a Gram-positive non-sporing bacillary organism, 2–3μ by 0·5μ (average), often in pairs end-to-end at an acute angle. It is feebly motile and possesses a single terminal flagellum. Sometimes elongated filaments may be observed.

Cultures can be obtained at 37° C. under aerobic conditions on ordinary media, but growth is better on media containing liver extract or glucose. The colonies are at first very small and droplet-like ; after a few days' growth they may attain a diameter of 2 mm., being smooth and transparent though later they may be more opaque. Gelatin is not liquefied. Glucose is regularly fermented, lactose and saccharose may be fermented, though slowly ; mannitol is not acted on.

When cultures are inoculated into rabbits and guinea-pigs, monocytosis results (*vide supra*), and if the dose is considerable, localisation may occur in the myocardium, meninges and liver with associated focal necrosis.

The species appears to be serologically somewhat heterogeneous and four types have been recognised by means of agglutination tests.

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[1] See Webb, R. A., and Barber, M., *J. Path. Bact.*, 1937, **45**, 523 ; and Wright, H. A., and Macgregor, A. R., *J. Path. Bact.*, 1939, **48**, 470.

CHAPTER XIII

THE TUBERCLE BACILLUS AND OTHER ACID-FAST BACILLI

BACILLUS TUBERCULOSIS (*Mycobacterium tuberculosis*)

The causative organism of tuberculosis in man, mammals and birds.

Under this designation are included different types —the "human," "bovine" and "avian" types, so called in virtue of their occurrence in man, cattle and birds respectively.

An acid-fast bacillus recently described in a tubercle-like disease among voles apparently represents a further type of tubercle bacillus.

HUMAN TYPE

Morphology.—Slender, straight or slightly curved rod-shaped organism, $2{\cdot}5$–$3{\cdot}5\mu$ by $0{\cdot}3\mu$, with rounded, pointed, or sometimes expanded ends. In the tissues they may occur singly, or in pairs often forming an obtuse angle, or in small bundles of parallel bacilli. The organism is non-motile, and non-sporing though it possesses considerable powers of resistance to drying. In old cultures individual cells may grow into long filaments and show branching.

Staining.—It is more difficult to stain than other bacteria. A strong dye with a mordant is required (*e.g.* carbol fuchsin—*vide* p. 203), and either prolonged staining or the application of heat. It may stain uniformly or show marked beading. When stained it resists decolorisation with 20–25 per cent. sulphuric or nitric acid, and also with alcohol, and is therefore described as "acid- and alcohol-fast." The tubercle bacillus is Gram-positive, but can be demonstrated only with difficulty by Gram's method. The "acid-fastness" has been attributed to lipoid substances in the organism, which can be dissolved out with hot alcohol or ether.

It seems likely that the tubercle bacillus may occur in the tissues in a non-acid-fast form which is not demonstrable by the Ziehl-Neelsen method. It was originally claimed by Much that a granular phase of the organism could be recognised, and recently it has been stated that a filterable form of the organism can be demonstrated. This still requires fuller confirmation.

Culture.—Aerobe; temperature range—30°–41° C. optimum—37°–38° C..

Does not grow on ordinary media. Primary growths may be obtained on serum media, on pieces of animal tissue, or on a medium containing egg yolk (*vide* p. 134). In secondary culture, growths may result on ordinary media (agar, broth, potato) with 5–6 per cent. glycerol added; growth is slow—*e.g.* ten days may elapse after primary inoculation, or even sub-inoculation, before growth is apparent.

The most convenient medium for artificial culture in ordinary laboratory work is one of the glycerol-egg media (*vide* pp. 134–136); the growth is luxuriant and presents the following appearance:—dry, irregular, tough and tenacious, wrinkled or mammillated, at first white, later buff-coloured.

In glycerol broth—growth consists of white flocculi forming a powdery deposit, but if a fragment of inoculum is floated on the surface, growth may spread on the surface of the medium as a white wrinkled pellicle.

Viability.—The thermal death-point is about 60° C.. While the majority of individual bacilli die when desiccated, a minority may survive for long periods. The organism is relatively resistant to injurious chemical substances: it can survive in putrefying material, it withstands the gastric juice, and *in* *sputum* resists 5 per cent. phenol and antiformin (*vide* p. 400) for several hours. It is highly susceptible to sunlight and ultra-violet radiation. Cultures remain viable usually for several months.

Animal Inoculation.—The guinea-pig is susceptible to experimental infection. If injected subcutaneously with the bacilli either in pathological material or in culture, after a few days a local swelling results consisting of tubercle nodules, which become confluent, undergo caseation and finally ulcerate. The neighbouring lymph glands become involved by spread of the bacilli along lymphatic channels and, later, lymph glands in other parts of the body are affected, showing the characteristic tuberculous lesions. The animal begins to lose weight, and dies in six weeks to three months. At autopsy, a general tuberculosis is noted; the spleen is enlarged and contains greyish-white tuberculous nodules or larger necrotic lesions. The liver and also various other organs present a similar condition. The lungs, however, may show relatively slight lesions and the kidneys may be practically free from tubercle nodules.

If the animal is killed four or five weeks after injection, tuberculous nodules may be present only in the spleen and on the peritoneum.

Animals can be infected also by inhalation and by feeding.

Occurrence in Animal Tuberculosis.—The human type of tubercle bacillus, apart from its occurrence in human disease, has been found also in tuberculosis of monkeys, pigs and dogs.

BOVINE TYPE

Morphology and *Staining* reactions are practically identical with those of the human type.

Culture.—As compared with the human type, growth is less luxuriant, and the bovine type is described as " dysgonic." On egg medium—it forms a thin, white, smooth, slightly moist, granular and easily broken-up growth (*cf.* human type—"eugonic"). The difference between the human and bovine types is accentuated by using a glycerol-egg medium. Glycerol favours the growth of the human type but has no such effect on the bovine variety.

Pathogenicity to Animals.—The bovine type is more virulent to cattle and laboratory animals than the human type. In the ox it produces a fatal tuberculosis, whereas the human type causes only a localised lesion which heals spontaneously.

The difference between the two types can be elicited by injecting a rabbit intravenously with an emulsion of 0·01–0·1 mgm. of dried bacilli (from a culture) in saline. The bovine type produces an acute generalised tuberculosis, and the animals usually die within two months; in the case of the human type the animals survive, or die only after two months, with slight lesions confined usually to the lungs and kidneys.

The differentiation may also be brought out by injecting 10 mgm. of culture subcutaneously in the rabbit; the bovine type leads to a general tuberculosis, which is fatal usually within ten weeks, whereas the human type produces only a local lesion.

It is to be noted that strains which deviate in their characters from the standard human and bovine types may be met with. Thus, strains isolated from lupus are frequently of attenuated virulence for laboratory animals (Griffith).

Recently it has been pointed out that voles are highly susceptible to the bovine type of tubercle bacillus whereas they are resistant to the human type; and it is suggested that a dose of 0·001 mgm. moist weight of culture injected intraperitoneally into voles will clearly distinguish between the two types.

The bovine type of tubercle bacillus, in addition to its association with tuberculosis of cattle, is the commonest variety found in tuberculosis of most other domesticated animals (*e.g.* pigs, horses, cats).

OCCURRENCE OF TUBERCLE BACILLI IN HUMAN LESIONS

Both the human and bovine types are met with, though the former is the more prevalent. The latter is infrequently found in adults, but a proportion of tuberculous conditions in young subjects is due to this type, particularly cases resulting from alimentary infection by milk from tuberculous cows.

The percentage frequency of the bovine type of tubercle bacillus, according to Griffith, is as follows: tuberculosis of cervical glands, 91·3 in England and 65 in Scotland in children under five years, and 50 in England and 52·6 in Scotland at all ages; bones and joints, 19·7 in England and 30·7 in Scotland; genito-urinary system, 17·4 in England and 31 in Scotland; lungs, 1·4 in England and 5·4 in Scotland; meningitis, 24·6 in England and 29·6 in Scotland; lupus, 48·7 in England and 69·2 in Scotland.

Tubercle bacilli are most numerous in acute lesions showing rapid caseation—*e.g.* acute phthisis. In acute miliary tuberculosis they appear to be relatively scanty. In chronic infections few tubercle bacilli are observed, and they may not be detectable microscopically though demonstrable by animal inoculation—*e.g.* in the pus from a tuberculous abscess. In the lesions they are usually found free from cells, but intracellular bacilli may be noted.

In phthisis, tubercle bacilli are present in the sputum, and often in large numbers if the pulmonary lesion is active and rapidly breaking down.

In tuberculosis of the urinary system the bacilli may be found in the urine by microscopic examination of the deposit after centrifuging, but as a general rule they are relatively scanty, and may not be observed in film preparations.

In intestinal tuberculosis the bacilli may in some cases be found in films from the faeces.

In tuberculous meningitis, tubercle bacilli may be seen in films from the spinal fluid after centrifuging, or in films from the coagulum which forms in the fluid after it is withdrawn. In early cases, however, microscopic examination may yield negative results.

Immunity.—Dead cultures of the tubercle bacillus have little immunising effect. A certain degree of resistance can be conferred, however, by introducing into the body living organisms of attenuated virulence. For this purpose Calmette and Guérin have advocated the use of a bovine strain attenuated by prolonged growth on a bile-glycerol-potato medium. This strain is generally designated "B.C.G." (Bacille Calmette-Guérin) and is practically non-pathogenic. It has been used as a vaccine for immunising children and cattle, and claims have been made that it is effective by oral administration in young subjects. The practical results are difficult to assess. It has been proved, however, by experiments in calves, that immunity follows intravenous injections of B.C.G., though of somewhat limited duration.

It may be noted that *experimental* immunisation against human and bovine types of the tubercle bacillus has been achieved by inoculation of living cultures of the vole type in animals to which this type is only slightly virulent (*e.g.* guinea-pig, calf).

DIAGNOSIS

In general, *direct microscopic examination* serves for ordinary diagnostic work.

Sputum—a film is prepared from the purulent portion of the sputum and stained by the Ziehl-Neelsen method (*vide* p. 204). A prolonged examination may be necessary in some cases where the bacilli are relatively scanty, and one examination with a negative result by no means excludes tuberculosis.

In young children who swallow their sputum, stomach contents obtained by gastric lavage may be examined, or coughing may be induced by inserting a swab into the throat, the secretion or expectoration on it being then used to prepare a film for microscopic examination. Repeated examination of faeces may also be resorted to as an alternative to examination of gastric contents.

Urine, pleural and peritoneal fluids—are centrifuged, films are made from the deposit and stained by the Ziehl-Neelsen method.

If a specimen of urine has not been taken with a catheter, it is essential to treat the film with alcohol (two minutes) after decolorisation with acid in the Ziehl-Neelsen process, in order to exclude smegma bacilli (*vide* p. 405). In examining urine it is advisable to obtain the sediment from a twenty-four hours' specimen and treat it with antiformin (*vide infra*).

Cerebro-spinal fluid—is allowed to stand in a stoppered tube for an hour or longer when a "spider-web" coagulum usually forms in the fluid. The clot is carefully decanted into a watch-glass, and a cigarette paper (held with forceps) is laid over it, gentle pressure being exerted with a wire loop so that the clot adheres to the paper on which it is then transferred to a slide. By firm blotting the clot is spread on the slide to which it remains adherent after the paper is removed. The preparation is dried and stained by the Ziehl-Neelsen method. In the absence of clotting the fluid is centrifuged and the deposit examined in the usual way.

Pus and faeces—films are made in the usual way, but it is advisable to treat with antiformin (*vide infra*).

In the case of tissues—sections are stained by the Ziehl-Neelsen method (*vide* p. 205).

Antiformin method for the detection of scanty tubercle bacilli in sputum, pus, tissues, etc.—By this method the bacilli can be concentrated in the material examined, and it is particularly valuable where the bacilli are scanty. The method is of course supplementary to the ordinary examination, and is quite unnecessary where bacilli can be seen in direct film, unless there should be any doubt as to the identity of acid-fast bacilli observed by the usual method.

"Antiformin" consists of equal parts of liquor sodae chlorinatae (B.P.) and 15 per cent. caustic soda. It has the property of dissolving cells and other bacteria, leaving tubercle bacilli intact.

A quantity of sputum or other material is treated with three or four times its bulk of antiformin diluted 1 in 6 with water, and the mixture is shaken and allowed to stand at 37° C. till it becomes thoroughly liquefied. This usually takes about an hour, but it is sometimes necessary to add more diluted antiformin to complete the solution. The mixture is then centrifuged and the supernatant fluid is removed. Water is added and mixed with the sediment. After centrifuging again, thick smears are made from the sediment, dried, fixed and stained by the Ziehl-Neelsen method.

Cultivation of Tubercle Bacilli from Pathological Material.—If the tubercle bacilli are likely to be present in pure culture in the material, tubes or screw-capped bottles containing slopes of egg medium can be inoculated directly. If other organisms are present—*e.g.* in sputum—the antiformin method or one of the procedures described below can be used and cultures made from the sediment; in this case the centrifuge tubes, water used for washing the sediment, etc., must be sterile.

Petroff's method (modified).—Sputum is mixed thoroughly with three to four times its volume of 4 per cent. caustic soda, and placed in the incubator at 37° C. for thirty minutes, the container being shaken from time to time. The mixture is centrifuged in a conical tube at 3000 r.p.m. for thirty minutes and the supernatant fluid poured off. The deposit is neutralised with 8 per cent. hydrochloric acid, which is added drop by drop, the reaction of the mixture being tested by adding a drop of phenol red solution to the tube. The deposit is then inoculated on egg medium containing 1 : 10,000 crystal violet (Petroff's medium), which inhibits the growth of other organisms, or preferably on the Löwenstein-Jensen medium (p. 135).

An alternative method is to treat with 6 per cent. sulphuric acid for the same time. In this case neutralisation of the centrifuged deposit with caustic soda is carried out before the culture medium is inoculated. In dealing with specimens of sputum some workers prefer Petroff's method in which tenacious mucus is dissolved by the alkali.

In applying the method of direct cultivation the possibility must be borne in mind of non-pathogenic acid-fast bacilli (*vide infra*) occurring in cultures from the various materials examined, these organisms sometimes yielding growths not unlike that of the tubercle bacillus. Careful scrutiny of the culture should therefore be made and if there is any doubt an animal inoculation test should be carried out.

A slower but certain method of obtaining pure cultures is to inoculate a guinea-pig with the material (p. 396), and to make cultures from the lesions in glands or internal organs (*e.g.* spleen) after active or generalised tuberculosis results. Cultures can be obtained from the inguinal glands if the lesions have not ulcerated through the skin. When this has occurred, cultures may be made from the pelvic glands.

In laboratory diagnosis, where tubercle bacilli cannot be detected in specimens by microscopic examination, *direct cultivation* or *guinea-pig inoculation* may be resorted to, and often yields positive results when microscopic examination is negative.

The usual method of carrying out the guinea-pig inoculation test is to inject material subcutaneously in the flank or thigh or intramuscularly in the latter situation. The result of the inoculation test can sometimes be expedited by intradermal inoculation, 0·4 c.c. of the material being injected into the shaved abdominal skin. If the specimen is tuberculous a nodule appears in 7–21 days ; this is incised and films of the lesion are examined for tubercle bacilli.

Tuberculin is a preparation containing the specific protein of the tubercle bacillus. It was originally prepared from a six-weeks-old culture in glycerol-broth, evaporated to one-tenth of its volume, sterilised by heat and filtered (Koch's "Old Tuberculin"). Various methods have been employed, however, in its preparation. Thus Koch's "New Tuberculin" is derived by grinding the bacilli (obtained from a growth on solid medium) in 50 per cent. glycerol. Tuberculin has also been prepared from cultures grown in a synthetic medium and has the advantage of being free from extraneous protein material which may give non-specific reactions. Moreover the specific protein can be separated from other constituents and products of culture and thus purified. The process of isolating this protein is a somewhat complicated one, involving ultra-filtration and precipitation by trichloracetic acid.

This "Purified Protein Derivative" (Tuberculin P.P.D.) is preferable to Old Tuberculin as it is constant in composition and potency. Moreover there is an absence of non-specific substances. It is issued in the dry state, from which it is easy to prepare dilutions by the addition of a borate buffer solvent. Tuberculin P.P.D. is used in the same way as, and replaces, Old Tuberculin.

It should be noted that tuberculins prepared from the human and bovine types of tubercle bacillus are indistinguishable by the usually accepted methods of standardisation as they contain the same specific substance.

Tuberculin has been used in the diagnosis and treatment of tuberculosis and for full details one of the larger works should be consulted. The diagnostic application depends on the fact that the tissues of a person or animal infected with tuberculosis may exhibit hypersensitiveness (allergy) to the specific protein of the bacillus. Thus, the subcutaneous injection of tuberculin in a tuberculous subject may lead to local, focal and general reactions. Cutaneous allergy can be elicited by the *cutaneous reaction of von Pirquet*, in which the "Old Tuberculin" is applied to a small abraded area of skin, or by the *intracutaneous test of Mantoux*, which consists in the intradermal injection of tuberculin (0·1 c.c. of a 1:1000 dilution) by means of a syringe with a fine needle. In both cases in a tuberculous subject an area of erythema and swelling appears at the site of inoculation within a few hours and attains a maximum in twenty-four to forty-eight hours. It is to be noted, however, that such reactions do not necessarily denote active tuberculosis and a high percentage of adults give a positive reaction probably due to previous sub-clinical infection. In infants and young children the reaction is of more significance from the diagnostic standpoint.

The tuberculin reactions have been utilised also in the recognition of tuberculosis in cattle, and are of great importance in testing milch cows. Thus, in England "tuberculin-tested milk," and in Scotland "certified milk" and "tuberculin-tested milk," must be obtained from tuberculin-tested animals which yield a negative reaction. The test in cattle is usually carried out in this country by the *double intradermal method*:

0·1 c.c. Old Tuberculin (or one of the newer preparations—*vide supra*) is injected intradermally in a shaved area of skin in the neck, and the thickness of a fold of skin in this area is measured with callipers before the injection. The result is observed after forty-eight hours and a positive reaction is indicated by the occurrence of a hot, tender, diffuse and ill-defined swelling, the increased thickness of the fold of skin being 7–17 mm.; in non-tuberculous animals there may be some degree of infiltration, which is usually well defined and small in area; if the result after the first injection seems negative or inconclusive, a second dose is given at the same site and the result noted after twenty-four hours. A positive reaction is indicated by the same type of inflammatory swelling as that described above, the increased thickness of the skin fold being stated as 13–40 mm.

Recently it has come to light that cattle may give positive reactions in the absence of demonstrable tuberculosis *post mortem*, possibly in certain cases as the result of inapparent infection by the avian type of tubercle bacillus or some other acid-fast organism.

By the Regulations under the Therapeutic Substances Act, the term tuberculin is restricted and applies to "preparations of fluid media in which *Bacillus tuberculosis* has been grown in artificial culture and which have been freed by filtration from the bacilli." These regulations stipulate that Old Tuberculin preparations shall be tested by an approved method for specific toxicity to guinea-pigs or other animals infected with the tubercle bacillus. A standard preparation of Old Tuberculin is kept in the National Institute for Medical Research, London, and Old Tuberculin shall not be issued if its activity differs from the standard preparation to such an extent that the difference is revealed by the test.

The *complement-fixation reaction* has been applied both in diagnosis and in gauging the activity of lesions. The technique has been referred to in Chapter VIII. The reaction is most frequent and most marked in the case of chronic but active lesions. It is negative in many arrested cases, and may be negative also in rapidly progressive tuberculous conditions.

AVIAN TUBERCLE BACILLUS.—The causative organism of a tubercle-like disease in birds. Its morphology and staining reactions are the same as those of the other types of tubercle bacilli. Its optimum temperature is 41°–42° C., and on glycerol-agar the growth is more rapid in development, moister, more homogeneous and more luxuriant than that of the mammalian types. Individual colonies are large, raised, hemispherical, with a smooth shiny surface and a yellow or brownish-yellow colour.

It is highly virulent to fowls, which are resistant to the mammalian tubercle bacilli. For testing purposes 0·001 mgm. is injected intravenously or the bacilli are administered by feeding. The guinea-pig, which is highly susceptible to the human and bovine types, is resistant to the avian bacillus.

This type of tubercle bacillus also occurs in pigs, and has been reported in other domesticated mammals including cattle. Human tuberculosis due to the avian type is extremely rare.

Acid-Fast Bacilli found in Cold-Blooded Animals.—Acid-fast bacilli resembling the tubercle bacillus have been isolated from fish, frogs, turtles, etc., and have been regarded as aetiologically associated with a tubercle-like disease in such animals. These organisms grow at low temperatures, even 15° C.. In cultural characters they correspond to the avian type (*vide supra*).

Acid-Fast Saprophytic Bacilli. — Non - pathogenic acid - fast bacilli may be found in milk, butter, manure and grass. They are similar in morphology to the tubercle bacillus, but their growth on culture medium is rapid ; they develop on ordinary media and at room temperature, producing an abundant dry or slightly moist growth which is irregular, coarsely granular and sometimes wrinkled ; most strains are definitely pigmented—yellow, pink or brown.

Acid-fast bacilli have also been demonstrated in water and may be found in the deposits from the interior of laboratory taps. This possibility must be borne in mind in using tap water for preparing films and staining solutions.

Smegma Bacillus.—This is a commensal organism found in the smegma and on the skin. It conforms in biolcgical characters to the saprophytic types described above. As it may occur in specimens of urine, it has to be differentiated carefully from the tubercle bacillus.

It is generally shorter and thicker than the latter, and shows greater variation in size and shape. The smegma bacillus is acid-fast, but in urinary deposits is usually decolorised by alcohol (*vide* p. 400), which has no effect on the tubercle bacillus.

BACILLUS LEPRAE (*Mycobacterium leprae*)

The causative organism of leprosy.

Morphology and Staining.—A straight or slightly curved slender bacillus, about the same size as the tubercle bacillus, with pointed, rounded or club-shaped ends ; so far as is known it is non-motile and non-sporing. Like the tubercle bacillus it requires as a rule a strong stain, and is acid-fast, though not to the same degree ; it may stain uniformly, but usually shows marked beading, which may be coarser than that of the tubercle bacillus ; it is Gram-positive, and can be stained fairly readily by the ordinary Gram's method (*cf.* tubercle bacillus).

Culture.—A great many attempts have been made by various workers to cultivate this organism ; the majority have been unsuccessful, and though successful results have been claimed and cultures of acid-fast bacilli have apparently been isolated from leprous lesions, it is doubtful whether these strains represent the true leprosy bacillus. The attempted cultivation of this organism is hardly within the scope of routine practical bacteriology, and for further information on this subject the text-books and other literature should be consulted.

Occurrence and Distribution.—Leprosy is an infective granuloma, developing as (1) the " nodular " type, in which nodules of granulation tissue form in the skin, mucous membranes and various organs (*e.g.* lungs, liver, spleen, testes), or (2) the " maculo-anaesthetic " type, where the granulation tissue infiltrates certain nerves and leads to motor and sensory paralysis with characteristic trophic changes (*e.g.* anaesthetic skin areas—" maculae "). Both types of the disease may occur in the same patient.

The organisms are found in the granulomatous lesions, being particularly numerous in the nodular form. They are distributed intracellularly for the most part, parallel bacilli occurring in bundles which may completely fill up cells. They may be found also in the tissue spaces, in the walls of small vessels, in skin glands, lymph glands, and in the secretions of the nose, throat and mouth, due to the fact that the mucosal lesions ulcerate readily and discharge bacilli into the mucous secretions. The organisms do not occur in the maculae which are essentially trophic and not primarily leprous lesions. In leprosy the bacilli have actually been observed in organs without associated lesions.

The bacilli are present in the nerve granulomata but are less numerous than in the nodular lesions.

DIAGNOSIS

Films are made from any ulcerated nodule on the skin, or a non-ulcerated nodule can be punctured with a needle and squeezed till lymph exudes, from which films are made. Films can be prepared also from a scraping of an excised piece of tissue, or sections may be prepared as for histological examination. A convenient method is to remove with curved scissors a piece of skin (about 2 mm. deep) overlying a nodule and prepare films from the deep surface.

The films or sections are stained by the Ziehl-Neelsen method, substituting 5 per cent. sulphuric acid for 20 per cent. The presence of the characteristic acid-fast bacilli, especially when they occur in large numbers and are situated inside cells, is diagnostic.

As a routine measure, films should be made in all cases from the nasal mucosa or secretion, as diagnostic information may be obtained in this way even when nodules are not present in the skin. This also applies to the maculo-anaesthetic cases.

When the lungs are affected the bacilli may be demonstrated in the sputum, but require to be differentiated from the tubercle bacillus by animal inoculation ; the leprosy bacilli do not produce any pathogenic effects in laboratory animals.

Bacillus (or *Mycobacterium*) *leprae murium*.—The organism of " rat leprosy " which presents some pathological similarity to human leprosy, is an acid-fast bacillus related to, but not identical with, *B. leprae*. This disease of rats is transmissible experimentally to animals of the same species, but not readily to other species, though transmission to the golden hamster has been recorded. It should be noted that human leprosy cannot be transmitted to rats.

BACILLUS OF JOHNE'S DISEASE (*Johne's bacillus ; Mycobacterium paratuberculosis*)

The causative organism of a chronic enteritis of cattle, and a similar disease of sheep.

Morphology.—A Gram-positive, acid-fast and alcohol-fast bacillus like the tubercle bacillus, but more readily stained by the Ziehl-Neelsen method. It is comparatively short (1 to 2u) and stains uniformly, though the longer forms may stain irregularly.

Culture. — Has proved difficult to cultivate artificially. Growths can be obtained on culture medium (*e.g.* glycerol-egg containing 1 per cent. killed *B. tuberculosis* or other acid-fast bacilli—e.g. *B. phlei*—or extracts of these organisms.[1]) An egg medium in which is incorporated a filtered glycerol-broth culture of the human tubercle bacillus forms a suitable substrate for the growth of this organism. After primary cultivation in this way subcultures may be obtained on egg media or glycerol-broth without the addition of another acid-fast organism or its products. Cultures tend to resemble those of the tubercle bacillus. On glycerol-broth Johne's bacillus may produce a wrinkled pellicle of growth like that of the tubercle bacillus. The optimum temperature is about 39° C..

Experimental Inoculation.—The disease is transmissible experimentally to calves, the incubation period being several months. Laboratory animals are insusceptible.

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[1] See Dunkin, G. W., *J. Compar. Path.*, 1928, **41**, 94.

Distribution.—The lesions are of a granulomatous nature and lead to corrugated thickening of the mucosa of the intestine ; the small bowel is primarily affected. The bacilli are present in large numbers, usually packed inside the cells of the lesion (as in leprosy).

Diagnosis.—At autopsy, the characteristic acid-fast bacilli may be demonstrated in the mucous membrane of the bowel by the appropriate staining methods.

During life, the organism may be observed in the excreta, especially if the antiformin method is used (*vide* p. 400). A diagnostic procedure which has been used in veterinary practice is to remove scrapings of rectal mucous membrane with a Volkmann's spoon, and from these to prepare films, which are stained by the Ziehl-Neelsen method. Tuberculosis may be excluded by the inoculation of material containing the acid-fast bacilli into laboratory animals.

An allergic skin reaction evoked by " Johnin," a preparation (from cultures) analogous to tuberculin, has been utilised in diagnosis, but its diagnostic specificity is doubtful.

CHAPTER XIV

BACILLUS MALLEI; BACILLUS ANTHRACIS AND BIOLOGICALLY ALLIED ORGANISMS

BACILLUS MALLEI (*Malleomyces mallei; Pfeifferella mallei*)

THE causative organism of glanders.

Morphology.—Straight or slightly curved bacilli with rounded ends, about 2–3μ by 0·4μ. Short forms are frequently noted and also longer filaments. Bacilli with club-shaped ends, and even branched forms have been observed. In old cultures swollen irregular involution forms are numerous. The bacilli occur singly or in pairs. They are non-motile and non-sporing.

Staining.—Gram-negative. An important feature of the organism, as seen in the tissues and inflammatory exudate, is its granular or beaded appearance.

Culture.—Aerobe and weakly facultative anaerobe; optimum temperature—35°–38° C.; does not grow below 20° C.; grows on ordinary media, but the addition of glycerol (4 per cent.) assists growth. Blood or serum also enriches cultures.

Agar stroke—uniform, white, semi-transparent, moist band of growth along needle track, which later becomes opaque, somewhat slimy and yellowish brown in colour.

Colonies on agar are about 1 mm. diameter after two to three days' growth; they are round and convex and have the same physical characters as the confluent growth described above. Colony variants presenting a dry and wrinkled appearance have been observed.

Glucose is fermented, but the reaction is slight and not regular in occurrence. A slight effect on salicin has been recorded. No other carbohydrates are fermented, but it is stated that growth in milk produces slight acidity and slow clotting.

Gelatin is not liquefied, but *B. mallei* does not grow well at room temperature.

Potato slope—at first a transparent yellowish growth ("honey-like"), later becoming opaque and of a chocolate-brown colour. Care should be taken to ensure that the potato is alkaline (*vide* p. 137).

In primary culture, growth does not occur readily and the organisms die out quickly, but after several subcultures they become adapted to a saprophytic existence, and may live for two months.

Occurrence.—Glanders is an infective granuloma, with a marked tendency, however, to suppurative change. It is essentially a disease of horses, asses and mules, and is only occasionally transmitted to man, usually by direct infection from an animal source.

In acute and subacute animal glanders, ulcerating nodules occur in the nasal mucosa and later in the lungs and internal organs. The bacilli are present in considerable numbers in all the lesions, situated for the most part extracellularly.

In chronic animal glanders ("farcy"), where the superficial lymph glands and vessels are involved, the bacilli are less numerous.

Latent infections are not infrequent in animals, and have also been observed in the human subject.

In human glanders, the infection usually originates in the skin (*e.g.* wound, abrasion, etc.), more rarely in the mucosa of the mouth or nose. The bacilli are found in the local inflammatory lesion and spread by the lymphatics, producing an acute lymphangitis. Ultimately a pyaemic condition results with secondary foci, in which the bacilli are numerous.

Animal Inoculation.—In equidae, the typical disease can be reproduced by subcutaneous injection of recently isolated cultures; asses are most susceptible.

Guinea-pigs are markedly susceptible, and after subcutaneous injection die in a week or two with generalised lesions, as in acute animal glanders. If a male guinea-pig is inoculated intraperitoneally, the tunica vaginalis is rapidly invaded, and externally swelling of the testis is noted (Straus reaction). Even when pathological material containing *B. mallei* in mixed infection is injected intraperitoneally, the specific organism seems to flourish best in the tunica, and can be isolated more easily from this situation than from the peritoneum.

DIAGNOSIS

Films are prepared from the pus, discharge from sores, etc., or from nodules in internal organs found at *post-mortem* ; these are stained with methylene blue and by Gram's method. The appearance of beaded Gram-negative organisms corresponding to *B. mallei* is suggestive. It should be noted that glanders bacilli are not easily demonstrable in preparations from the lesions.

Cultures are also made on glycerol-agar, and if a mixed growth results, pure cultures are obtained from single colonies. The chocolate-coloured growth on potato is an important criterion in identification.

In all cases the nature of the infection must be confirmed by animal inoculation. A male guinea-pig is injected intraperitoneally with the pathological material or the culture isolated ; in two or three days an enlargement of the testis results, and the animal subsequently dies, showing the lesions of acute glanders (*vide supra*). If pyogenic organisms are also present in the material injected, and a septic peritonitis results, the glanders bacilli will be found more numerous in the tunica vaginalis. If the inoculum contains a large number of other organisms it may be introduced by subcutaneous injection. The organism can then be recovered from the enlarged regional glands and tested further by intraperitoneal injection of pure cultures.

Agglutination Test.—In the diagnosis of glanders in horses, the agglutination test has been used. The technique followed is that described on pp. 250–254, with certain minor differences. The culture selected should be one which has been proved suitable for the agglutination reaction by previous tests with positively-reacting sera. The bacterial suspension is prepared from young glycerol-agar slope cultures, and is then heated at 65° C. for one hour to kill the organisms. For personal safety it is necessary to use dead cultures. Serum dilutions ranging from 1 in 100 to 1 in 4000 are tested. The agglutination tubes are incubated for two hours at 37° C. and are then kept in a refrigerator overnight before readings are made. Normal horse serum, however, may agglutinate the bacillus, and only agglutination in high dilutions, 1 in 1000 or more, can be accepted as proof of infection. Agglutination in lower titres does not, of course, exclude the existence of the infection. Any spontaneous agglutination in the control would, of course, invalidate the result. Some workers include parallel tests with normal horse serum as an additional control.

A *complement-fixation test* (*vide* pp. 43, 270) has also been employed as a serum diagnostic method. It has been regarded as more satisfactory than the agglutination test, especially in chronic cases.

The antigen is prepared in the same way as the bacterial suspension for the agglutination test. The concentration to be used in the actual test should be free from anti-complementary action. This can be determined by a preliminary titration, varying dilutions being mixed with 2 M.H.D. of complement and incubated for one-and-a-half hours at 37° C. when the haemolytic system is added. After further incubation for one hour, the lowest dilution which exhibits no anti-complementary action (*i.e.* complete lysis) is selected for the complement-fixation test.

Mallein is a preparation analogous to tuberculin. A four-weeks' culture in glycerol-broth is sterilised at 100° C., and then filtered. The filtrate which contains the heat-stable soluble products of the organism is concentrated to one-tenth of the original volume and constitutes the preparation, "crude mallein," which can be readily preserved and utilised for diagnostic purposes in a manner analogous to tuberculin. Subcutaneous injection of 1 c.c. diluted mallein (containing 0·1 c.c. of the crude preparation) in an infected animal produces a local reaction (a swelling reaching a diameter of five inches after twenty-four hours) and a general reaction manifested by a definite elevation of temperature. A conjunctival reaction may be elicited readily by introducing mallein into the conjunctival sac, and is a more satisfactory test than that in which the mallein is injected subcutaneously. Another method which has been employed is the *intradermal-palpebral test*, in which 0·1 c.c. of crude mallein diluted 1 in 4 is injected intradermally near the margin of the lower eyelid. A pronounced swelling of the eyelid with conjunctivitis continuing for three or four days constitutes a positive reaction.

BACILLUS WHITMORI (*Malleomyces pseudomallei ; Pfeifferella whitmori*)

The causative organism of Melioidosis—a glanders-like disease occurring in the Malay States where it is epizootic among rodents, *e.g.* rats, and from these animals transmissible to man. The disease has been reported also in Cochin-China and Ceylon. The organism is similar to *B. mallei*, but is motile and grows well in gelatin at 20° C., liquefying the medium. Mucoid and corrugated types of growth on agar have been described and a brown growth on potato similar to that of *B. mallei*. Glucose, lactose, dulcitol, saccharose and mannitol are fermented (without gas production). Susceptible animals (*e.g.* guinea-pig, rat) may be infected experimentally. In the male guinea-pig the Straus reaction occurs as in animals infected with *B. mallei*. *B. mallei* and *B. whitmori* are serologically related.

BACILLUS ANTHRACIS

The causative organism of anthrax in animals and man.

Morphology.—A non-motile, straight, rod-shaped, sporing bacterium, rectangular in shape and of relatively large size—4–8μ by 1–1·5μ. The bacilli tend to be arranged in chains end to end (streptobacillus), but may occur singly and in pairs ; in blood and tissue they exhibit a distinct capsule when suitably stained. The spore appears first as a small refractile body in the centre of the bacillus, and appears to increase in size till it can be seen as an oval structure, central in position and of the same cross-diameter as that of the bacillus. Sporulation occurs readily when the organism is discharged from the body of an infected animal, and spores are a morphological feature of the bacilli when growing in artificial culture, but *sporulation does not occur in the tissues.* After the spore is fully formed the residual protoplasm of the bacillus disintegrates and the spore becomes a free structure. The spore represents a highly resistant phase of the organism, and can survive under conditions which would be unfavourable to the vegetative form. When replaced in favourable conditions, the spore capsule ruptures at one pole and the vegetative phase is reproduced.

Staining.—Gram-positive. The spore is unstained by the ordinary methods, but can be stained differentially by special methods (*vide* p. 208).

Methylene-Blue Reaction of McFadyean.—This staining reaction has been utilised in veterinary work for the recognition of anthrax bacilli in blood films. The films are made in the usual way on slides, dried and passed rapidly three times through the flame ; they are then stained with polychrome methylene blue for a few seconds (*vide* p. 195), washed and dried. Between the bacteria an amorphous purplish material is noted, representing the disintegrated capsules of the organisms ; this appearance is characteristic of the anthrax bacillus.

Culture.—Aerobe and facultative anaerobe ; temperature range—12°–45° C., optimum—35° C. ; grows on all ordinary media; aerobic conditions are necessary for sporulation, for which the optimum temperature is 25°–30° C..

Colonies on agar—white, granular, circular disks (about 3 mm. in diameter) which, under the low power of the microscope, show a wavy margin, often likened to locks of hair. The colony is one continuous convoluted thread of bacilli in chain formation.

Agar stroke—thick, white, opaque, somewhat dry, friable growth with notched edges, showing the same microscopic characters as the colonies. To the naked eye this growth presents a "ground-glass" appearance.

Gelatin stab—a line of growth along the needle puncture, from which fine lateral spikes radiate—longest towards the top. This is the so-called "inverted fir-tree growth"; liquefaction occurs later, starting at the top of the growth.

Coagulated serum is partially liquefied.

In broth—growth develops as white flakes which sediment, and sometimes shows pellicle formation.

Growing on blood-agar the anthrax bacillus is only slightly haemolytic as compared with the "anthracoid" bacilli (*vide* p. 422) which are markedly lytic.

Glucose, saccharose and maltose are fermented (without gas production).

Variation.—Capsule formation is subject to variation, and when the capsule is absent or imperfectly developed the colonies tend to be moist and slimy and may be devoid of the characteristic wreathed margins. This is well seen in cultures which have been attenuated in virulence by growth at temperatures above the optimum (*e.g.* 42°–43° C.) as in Pasteur's method of attenuating the organism for prophylactic vaccination.

The typical colony, as described, is of the "rough" form: the variant is small, "smooth" and without the characteristic wreathed appearance, while the bacilli in this type of colony are arranged in bundles, not in a convoluted chain. Virulence is associated with the "rough" form, the "smooth" variant being relatively avirulent.

Viability.—The thermal death-point of the vegetative form is about 60° C.. The spores can withstand 100° C. (moist heat) for five to ten minutes, and resist desiccation for an indefinite period. They are destroyed by 4 per cent. potassium permanganate in fifteen minutes. For disinfection of wool a 2 per cent. formaldehyde solution is used and allowed to act for twenty minutes at 102°–105° F. (39°–40·5° C.).

Occurrence in Animal Lesions.—The anthrax bacillus produces an epizootic disease in herbivorous animals, particularly among sheep and cattle. The condition is usually septicaemic in nature, and, *post mortem*, the bacilli are found in large numbers in the heart blood and internal organs, especially in the *spleen*, which is enlarged, soft and diffluent.

Occurrence in Human Lesions.—Transmission of the infection to man is from an animal source :

(1) Infection may occur through the skin—*e.g.* in persons handling infected animals, carcases or hides, from shaving brushes, etc.—the resulting lesion being described as a " malignant pustule ", *i.e.* an area of intense inflammation, with a central slough and with surrounding inflammatory oedema. Lymphatic spread may occur, and even septicaemia.

(2) Infection may result from inhalation of spores carried in dust or filaments of wool from infected animals, as in the wool factories—" wool-sorters' disease." The organisms settle in the lower part of the trachea or in a large bronchus, and an intense inflammatory lesion results, with haemorrhage, oedema, spread to the thoracic glands, involvement of the lungs, and effusion into the pericardial and pleural cavities ; the organisms are present in considerable numbers in the lesions ; a septicaemic condition may also supervene.

(3) Infection may occur by the intestine, but this is relatively uncommon in man.

Infection in Animals.—Animals may be infected from pasture which has become contaminated with *B. anthracis* spores derived from previous cases of the disease in the particular area. The disease is also spread by artificial foodstuffs—*e.g.* oil-cake. Biting flies—e.g. *Stomoxys*—may convey the organism, though in an entirely mechanical way.

Experimental Inoculation.—Guinea-pigs and white mice are most susceptible. If a guinea-pig is injected subcutaneously with pathological material containing the bacilli, or with cultures, the animal dies usually within two days, showing a marked inflammatory lesion at the site of inoculation and extensive gelatinous oedema in the subcutaneous tissues. Large numbers of bacilli are present in the local lesion. The animal shows a profound septicaemia, and the anthrax bacilli are present in large numbers in the heart blood and in the capillaries of internal organs. They are *specially numerous in the spleen*, which is enlarged and soft.

Prophylactic Immunisation.—Pasteur's vaccine has been extensively applied in the prevention of the disease among animals. The vaccine is a culture of the bacillus attenuated by growing at 42°–43° C.. The degree of attenuation is, however, difficult to regulate. Besredka has advocated the intradermal inoculation of the vaccine (as opposed to the subcutaneous injection of the older method) and claims that a solid immunity can be produced in this way. A " combined " method of prophylactic immunisation has also been applied—*viz.* injection of Pasteur's vaccine along with an immune serum.

Therapeutic Antiserum.—The serum of artificially immunised animals—*e.g.* Sclavo's serum—is used in the treatment of human anthrax, and is able to confer passive immunity. 50–100 c.c. doses are given intravenously and repeated daily if necessary.

DIAGNOSIS

Malignant Pustule—

1. Films are made from the exudate and stained by Gram's method ; the finding of bacilli morphologically like *B. anthracis* is suggestive but not conclusive. If there are unbroken vesicles round the lesion, fluid from these should be examined.

2. Successive-stroke inoculations should be made on an agar plate. The resulting colonies are recognised by examining them with the low power of the microscope, and films are made and stained by Gram's method. Spores are noted in cultures.

3. In all cases the identity of the suspected organism must be confirmed by inoculation of a

guinea-pig or mouse with exudate from the lesion, or with the isolated culture. A small dose of culture is sufficient to produce a lethal effect. The occurrence of the bacilli in the heart blood and in the spleen in considerable numbers, and the other *post mortem* appearances described above, are diagnostic.

If exudate or other material used for inoculation contains other organisms it is advisable to inoculate it on a scarified area of skin in preference to subcutaneous injection.

Diagnosis of Anthrax in Domestic Animals (*post mortem*).—The usual form of *post mortem* examination must not be made—*i.e.* to prevent any distribution of sporing bacilli from the carcase. In the body no sporulation occurs, but spores are readily formed when the bacilli are exposed to air. A film of blood taken from a superficial vein in the ear is prepared, and stained by Gram's method and by McFadyean's methylene blue method (*vide supra*). The finding of characteristic bacilli in the blood giving the methylene blue reaction is diagnostic. In pigs and horses the bacilli may not be detectable in the blood. If necessary, the organism can be cultured and identified by the procedure described above, a specimen of blood from the ear being used for the investigation.

Precipitin Test.—This test was first used by Ascoli in the recognition of anthrax infection in organs and tissues from suspected carcases, and may be applicable even in the case of putrefied material. It depends on the occurrence of a specific precipitin (*vide* p. 43) in the serum of an artificially immunised animal. Immune sera, however, vary in their precipitin content, and for the test a serum with known precipitating properties must be selected. About 2 grams of the tissue are boiled for five minutes with 5 c.c. of normal saline, to which acetic acid has been added in the proportion of 1:1000. The fluid is cooled and then filtered through paper. 0·5 c.c. of the serum is placed in a narrow tube and the filtrate is carefully run on to the top. The development of a white ring of precipitate at the junction of the two fluids within fifteen minutes denotes a positive result.

Examination of Shaving Brushes, Wool, etc., for B. anthracis

The bristles or wool are cut up with scissors into small fragments and shaken thoroughly with several volumes of sterile salt solution or soaked in a 3 per cent. solution of caustic potash so as to obtain " washings " from the material. The fluid is decanted and centrifuged. The sediment is suspended in about 1 c.c. of saline and heated at 65° C. for five minutes to eliminate non-sporing organisms present. (It is inadvisable to heat at higher temperatures or for longer periods as *B. anthracis* spores may not survive under these conditions.) Cultures on agar plates are then made from the suspended sediment, and guinea-pigs are injected subcutaneously with the material, and later with cultures of any suspected organism isolated if the direct inoculation test is negative. It is to be noted that in such examinations, organisms very similar in their morphological and cultural characters to the anthrax bacillus may be encountered—*e.g.* " *B. anthracoides* " (*vide infra*). Such organisms, if injected in large doses, may also produce fatal effects in mice and guinea-pigs. Their differentiation from *B. anthracis* is referred to later.

THE AEROBIC GRAM-POSITIVE SPORING BACILLI BIOLOGICALLY ALLIED TO BACILLUS ANTHRACIS

These organisms are saprophytes and represent a large number of different species. They are found in soil, water, dust and air. Being ubiquitous, they are frequent contaminants of culture medium in the laboratory, and bacteriological workers should be acquainted with their general biological characters.

Classical types representative of this group are *B. subtilis* (the " Hay bacillus "), *B. mycoides*, *B. mesentericus*, *B. megatherium*, *B. anthracoides*. The type-species is *B. subtilis*, and for convenience these organisms are sometimes spoken of as the " *B. subtilis group*." For the detailed differential features of the various species, reference can be made to *Bergey's*

Manual of Determinative Bacteriology (5th edition). The general characters of the commoner types met with in laboratory work may be summarised as follows, and for general purposes it is unnecessary to identify a particular species :—

Morphology.—Certain types tend to resemble the anthrax bacillus and young forms are Gram-positive. Some are morphologically identical with *B. anthracis* and occur in similar chain formation. Others are shorter with rounded ends, and several motile species with peritrichous flagella are met with (e.g. *B. subtilis*). The spore is central or excentric (e.g. *B. subtilis*, *B. mycoides*), subterminal or terminal. It may be relatively small, not exceeding 0·8μ (e.g. *B. mesentericus*) or large, up to 1·8μ (e.g. *B. megatherium*).

Culture. — The optimum temperature is usually low—*e.g.* about 20° C.—but certain types grow best between 30° and 37° C. and some are thermophile with their optimum temperature at 55° C. ; they are characteristic aerobes but usually also facultative anaerobes ; abundant growth occurs on all the ordinary culture media. The appearances of growths vary considerably among different types. *B. subtilis* produces a white, glistening, adherent, somewhat membranous growth, which tends to spread, and somewhat similar growths are seen among other species. Certain types produce colonies and growths practically similar to *B. anthracis*, with the same " wreathed " appearance of the margins—e.g. " *B. anthracoides.*" The colonies of *B. mycoides* are at first similar to those of the anthrax bacillus, but are easily differentiated by their feathery appearance due to long projecting and branching threads radiating out from the central growth. The growths may be dry, gummy or moist, and white, greyish white, yellowish or brown. Certain species producing a black pigment have been described. On potato, characteristic cultural appearances may be noted

— e.g. *B. mesentericus* develops a thick wrinkled or folded layer of growth which assumes a brownish colour. Pellicle formation on broth is a frequent character. Generally gelatin is liquefied and proteolytic action is well developed. Some types ferment carbohydrates. Starch may be hydrolysed.

These organisms are usually non-pathogenic on experimental inoculation into laboratory animals.

B. anthracoides.—This designation has been applied to a type of organism of the *B. subtilis* group which in morphological and cultural characters closely resembles *B. anthracis* and may exhibit colonies with the " wreathed " margins characteristic of the latter. Under certain conditions this organism might at first be confused with the anthrax bacillus. It can be differentiated usually, however, by its motility. It is to be noted that if a large dose of culture of this type of organism is injected into a guinea-pig or white mouse, a local inflammatory lesion with inflammatory oedema may be produced and septicaemia with a lethal effect. The organism can be detected in the heart blood and internal organs, though *in small number* (*cf.* anthrax). In blood or tissues it does not exhibit the McFadyean methylene blue reaction (*vide* p. 419).

CHAPTER XV

THE GRAM-NEGATIVE AEROBIC BACILLI OCCURRING AS COMMENSALS OR PATHOGENS IN THE INTESTINE; ENDAMOEBA HISTOLYTICA AND OTHER INTESTINAL PROTOZOA; LACTOBACILLI

THE GRAM-NEGATIVE INTESTINAL BACILLI

THESE organisms comprise the following main groups :—

1. *B. coli* group.
2. Typhoid-paratyphoid or " enteric fever " group.
3. Food-poisoning group.
4. Dysentery group.

The basis of this classification is clinical rather than biological—*e.g.* the food-poisoning organisms are closely related biologically to the typhoid-paratyphoid bacilli and all these organisms are frequently spoken of as the *Salmonella* group.

General Characters.—Gram - negative, non - sporing bacilli, about 2–4μ by 0·5μ (average)—aerobes and facultative anaerobes—growing best about 37° C.—fermenting various carbohydrates but not usually liquefying gelatin or coagulated serum.

The various groups and species are differentiated by cultural, biochemical and serological tests.

BACILLUS COLI GROUP (*Escherichia*)

This group includes a considerable number of different types, generally designated by the generic term *B. coli* or "coliform bacilli," occurring normally in the large intestine of man and other mammals.

Morphology and Staining.—Gram-negative bacilli, 2–4μ by 0·5μ, but filamentous forms up to 8–10μ in length may occur, and short cocco-bacillary forms are not infrequent. Types vary in motility. The motile varieties show a peritrichous arrangement of their flagella. Some forms possess a mucoid capsule.

Culture.—Aerobe and facultative anaerobe. Temperature range—10°–45° C., optimum—37° C.. Grows abundantly on ordinary media.

Colonies on agar—relatively large, thick, greyish-white, moist, circular disks ; the opacity and size vary with different strains ; capsulate strains produce colonies and growth of a viscid or mucoid consistence ; the margin of the colonies may be circular or wavy. "Rough" colony variants may be observed.

Gelatin stab—a white line of growth develops along the needle track, with a disk at the top on the surface of the medium ; gas spaces form in the gelatin due to the fermentation of the natural muscle sugar of the meat infusion which is the basis of the medium ; no liquefaction occurs unless in the case of certain atypical forms, e.g. *B. cloacae.*

In broth—a uniform turbidity usually results.

Potato slope—white moist growth, later assuming a brownish colour.

Biochemical Reactions.—The most prevalent types of *B. coli* in the intestine ("typical" or "faecal" *B. coli*) exhibit the following reactions :—

Glucose	*Lactose*	*Dulcitol*	*Saccharose*	*Adonitol*	*Inositol*	*Indole*	*Voges-Proskauer reaction*	*Methyl-red reaction*	*Citrate utilisation*
+	+	±	±	−	−	+ (*vide infra*)	− (*vide infra*)	+ (*vide infra*)	− (*vide infra*)

Motility ±

(*Vide* p. 121 for method of testing, and Table on p. 470*a*.)

The *B. coli communis* (Escherich)—*Escherichia communis*—one of the commonest of these types, has the following characters :—

+	+	+	−	−	−	+	−	+	−

Motility +

(+ under various carbohydrates signifies acid and gas formation.)

Indole Production.—This is tested for by growing the organism in peptone water, and after two days withdrawing with a sterile pipette 2 or 3 c.c. into a test-tube. An equal volume of Ehrlich's rosindole reagent is then added :

Para-dimethyl-amino-benzaldehyde	4 grams
Absolute alcohol	380 c.c.
Pure hydrochloric acid	80 c.c.

A rose colour develops in the presence of indole, and can be separated out with amyl alcohol. The addition of a saturated solution of potassium persulphate hastens the reaction. If the indole reaction is negative after two days' growth, the test should also be repeated after seven to ten days, as some strains are slow in their production of indole.

An alternative procedure is to add about 1 c.c. of ether to the culture which is then vigorously shaken. The ether extracts the indole and after it has separated, the culture having been allowed to stand for a few minutes, about 0·5 c.c. of Ehrlich's reagent is added.

Voges-Proskauer Reaction.—This reaction is exhibited by certain atypical varieties of *B. coli.* About 2 c.c. of a 10 per cent. solution of caustic potash are added to 10 c.c. of a two days' glucose-broth culture, and the culture is then allowed to stand at room temperature for some hours. If the reaction is positive an eosin-like colour develops, due to a reaction between diacetyl (formed by oxidation of acetyl-methyl-carbinol) and a guanidine residue in the peptone.

This test can be rendered more delicate by adding a trace of creatine to the culture and 5 c.c. 40 per cent. sodium hydroxide. Within a few minutes a pink colour develops if the reaction is positive (O'Meara).

Methyl-red Reaction.—This reaction indicates the hydrogen-ion concentration produced by growth in a standard glucose medium. The organism is grown for three days in a 0·5 per cent. peptone-water medium containing 0·5 per cent. glucose and 0·5 per cent. di-potassium hydrogen phosphate.

(Part of this culture may be used for the Voges-Proskauer reaction.) To approximately 10 c.c. of culture two drops of a methyl-red solution are then added (0·1 gram methyl-red dissolved in 300 c.c. alcohol and then made up to 500 c.c. with distilled water). A *red* colour denoting a high hydrogen-ion concentration (pH 4·5 or less) is described as "*positive*"; *yellow*, signifying a low concentration, is "*negative*." The prevalent type of *B. coli* in excreta is "methyl-red positive."

Citrate-utilisation Reaction.—This test has also been used for separating typical and atypical forms of *B. coli* in the examination of water supplies. It depends on the ability of such coliform bacilli as *B. lactis aerogenes* to utilise citrate as a source of carbon[1] and to grow in a synthetic medium containing this salt, the typical forms being unable to grow under these conditions. The medium recommended by Koser[2] consists of 1·5 grams sodium ammonium hydrogen phosphate (microcosmic salt), 1 gram potassium di-hydrogen phosphate, 0·2 gram magnesium sulphate and 2 grams sodium citrate in 1000 c.c. distilled water. *B. aerogenes* types grow and produce a visible turbidity in the medium.

Other Biochemical Reactions common to the typical B. coli.—On MacConkey's bile-salt neutral-red lactose agar—the colonies are rose-coloured, due to the action on the neutral red of the acid produced from the lactose.

In litmus milk—acid and clot produced.

In fluid media containing neutral red—a green fluorescence may develop, especially under anaerobic conditions.

In media containing nitrates—nitrates are reduced to nitrites.

Carbon dioxide and hydrogen are produced by the fermentation of glucose in the following proportions: $CO_2 : H_2$—1: 1–2.

Eijkman Test.—Typical strains of *B. coli* produce gas in a lactose medium when growing at 44° C.; some atypical forms exhibit no gas production, and this difference has been used as a criterion in distinguishing "faecal" from "non-faecal" strains (p. 299).

Haemolysis.—Some strains, particularly those isolated from urinary infections, are haemolytic towards human red cells. This property can be elicited best by growing the organism in a fluid medium to which blood has been added.

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[1] See Brown, H. C., *Lancet*, 1921, i, 22.

[2] Koser, S. A., *J. Bacteriol.*, 1923, **8**, 493; and *Ibid.*, **9**, 59.

ATYPICAL VARIETIES OF B. COLI

(1) *B. aerogenes* subgroup (*Aerobacter*). The so-called *B. lactis aerogenes* (*Aerobacter aerogenes*) belongs to this subgroup. These organisms ferment inositol and adonitol, and usually exhibit the Voges-Proskauer reaction. They are non-motile and are characterised by their large, raised, slimy or viscid colonies. The citrate-utilisation reaction is positive, the organism growing in Koser's citrate solution (*vide* p. 426). Indole production is usually absent and the methyl-red reaction is negative. The $CO_2 : H_2$ ratio (*vide supra*) may differ from that of the typical *B. coli*, being approximately **2 : 1**.

(2) Atypical varieties are met with, which in bio-chemical reactions seem to occupy an intermediate position between *B. aerogenes* and the typical forms as defined above. Among these are strains (*Escherichia freundii*) which differ from the typical *B. coli* in utilisation of citrate, adonitol fermentation or absence of indole production. These are less frequently found in faeces than the typical forms and some are only occasionally observed.

(3) Certain non-lactose-fermenters. Some of these may ferment lactose after spontaneous variation in culture (*vide* Table, p. 470*a*).

(4) Non-gas-producing types are occasionally met with—*B. coli anaerogenes* (*vide* Table, p. 470*a*).

B. cloacae (*Aerobacter cloacae*).—This organism is generally regarded as closely related to the *B. coli* group, but differs in respect of its liquefaction of gelatin (*vide* Table on p. 470*a*). In other characters it tends to resemble *B. aerogenes*. It has been found in sewage and polluted water.

The *B. coli Test in Water Examination* is referred to on p. 296.

Occurrence of B. coli Group.—Reference has already been made to their occurrence normally in the intestinal contents, where they are present in very large numbers. These organisms are also potential pathogens. They are found most frequently in pyogenic infections of the urinary tract (pyelitis, cystitis, etc.) either in pure culture or mixed with pyogenic cocci, cholecystitis and cholangitis, appendix abscess, peritonitis.

DIAGNOSIS

In film preparations from pus, urinary sediment, etc., stained by Gram's method, *B. coli* can be recognised as a Gram-negative bacillus of the average dimensions described above, but the morphological characters of this organism are similar to those of many other related species. If cultures are made on a differential medium containing lactose and an indicator of acidity, their identity can be established at once *in the case of the typical forms*, by the characteristic colour change in the indicator, *e.g.*, on MacConkey's medium by the rose-pink colour of the colonies. It must be remembered, however, that certain strains of *B. coli*, even those which ferment lactose in fluid medium, may at first produce pale colonies on MacConkey's medium, so that for identification it may be necessary to isolate the strain and test its reactions in detail.

BACILLUS OF FRIEDLÄNDER (PNEUMOBACILLUS)
(*Klebsiella pneumoniae*)

Originally described as a causative organism of pneumonia.

Morphology and Staining.—A small non-motile, non-sporing, Gram-negative bacillus with rounded ends and varying greatly in size—1–4μ by 0·5–1μ. The shorter forms simulate cocci. The bacilli occur usually in pairs, but also singly, and in short chains. They are typically capsulate, especially when seen in the tissues.

Cultures.—In cultural characters it generally resembles the *aerogenes* subgroup of coliform bacilli. The colonies exhibit a characteristic mucoid or viscid consistence, already referred to on p. 424.

Biochemical Reactions.—Different strains vary. Some correspond closely with *B. aerogenes* (*vide* Table, p. 470*a*). Others are non-lactose fermenters. Some strains form gas slowly, others apparently fail to produce gas in fermentation tests. With the majority of strains the indole reaction is negative and the methyl-red reaction positive. The Voges-Proskauer reaction is in some cases positive.

This organism may be regarded as biologically related to the *B. coli* group, though its habitat as a commensal or pathogen is generally in the upper respiratory passages.

Occurrence in Human Disease.—Friedländer's bacillus is only rarely responsible for cases of lobar pneumonia. It may be associated, however, with catarrhal conditions of the respiratory tract, suppuration in nasal sinuses, meningitis, conjunctivitis, empyema, etc..

Serological Classification.—Three serological types have been defined (analogous to the types of pneumococcus) ; a certain proportion of strains, however, cannot be identified with these types and forms a serologically heterogeneous group analogous to Group IV of the pneumococcus. Type-specificity depends on a carbohydrate substance contained in the bacterial capsule. It is of special interest that the specific substance of one of these types has been identified serologically with the specific substance of Type II pneumococcus.

BACILLUS OF RHINOSCLEROMA (*Klebsiella rhinoscleromatis*).—This organism closely resembles Friedländer's bacillus in morphology and cultural characters, but produces no gas from glucose, and does not ferment lactose. It does not grow on media containing bile and does not give the indole and Voges-Proskauer reactions. It is associated with a chronic granuloma of the mucous membrane of the nose, mouth or throat. The bacilli can be seen in the lesions, situated intracellularly. The disease is prevalent only in South-Eastern Europe.

BACILLUS OZAENAE (*Klebsiella ozaenae*).—Closely resembles Friedländer's bacillus, but is non-gas-producing in glucose. It is found associated with ozaena, but its aetiological relationship is doubtful.

TYPHOID-PARATYPHOID OR ENTERIC FEVER GROUP

This group includes :

B. typhosus
B. paratyphosus A
B. paratyphosus B

An organism with similarities to *B. paratyphosus A* and *B* has also been noted in certain cases of enteric fever, and designated *B. paratyphosus C* (*vide* p. 444). Certain other types belonging to the *Salmonella* group have occasionally been reported in cases of enteric fever (Table, p. 450).

It is convenient to classify the typhoid and paratyphoid infections together as " enteric fever."

BACILLUS TYPHOSUS (*Eberthella typhosa*)

The causative organism of typhoid fever.

Morphology and Staining.—A Gram-negative non-sporing bacillus, about 2–4μ by 0·5μ, actively motile, with numerous long flagella (peritrichous) ; longer filamentous forms are common.

Culture.—Aerobe and facultative anaerobe ; temperature range, 15°–41° C. ; optimum temperature 37° C.. Grows well on ordinary media.

Colonies on agar—like those of *B. coli*, but smaller, thinner and more transparent ; stock laboratory cultures may show a mixture of " smooth " and " rough " colonies (*vide* p. 42).

Gelatin stab—like *B. coli*, but the growth is less abundant, and there is no gas formation. No liquefaction occurs.

Colonies on MacConkey's medium — smaller than *B. coli* and " pale " or colourless, *B. typhosus* being a non-lactose-fermenter.

Desoxycholate-citrate medium—colonies also " pale " or colourless (as compared with *B. coli.*).

Biochemical Reactions.—Ferments glucose and mannitol with acid but no gas formation ; does not ferment lactose or saccharose and does not produce indole. Some strains exhibit slow fermentation of dulcitol. (See Table on p. 470*a*.)

Viability.—The thermal death-point is about 56° C.. The majority of individual bacilli die within a few hours when subjected to drying. In water the bacilli gradually die, but may survive for some time. Thus, in sewage-polluted sea- and fresh-water viable bacilli have been found after four weeks; in soil survival may occur after six weeks or longer.

Occurrence.—This organism shows a selection for lymphoid tissue — *e.g.* the Peyer's patches and lymphoid follicles of the intestine, the mesenteric glands and spleen. Thus, it attacks the Peyer's patches of the small intestine, and leads to an acute inflammatory reaction and infiltration with mononuclear cells, followed by necrosis, sloughing and the formation of the characteristic typhoid ulcers. It is present in large numbers in the inflamed tissue, in the ulcers, and is found in the intestinal contents and the dejecta. The mesenteric glands show similar inflammatory changes. At an early stage of the disease—during the first week or ten days—and in relapses, a condition of bacteraemia exists, and the typhoid bacillus can be demonstrated by blood culture. It is also present in the spleen, occurring in clumps. The bacillus is frequently present in large number in the gall bladder. It may localise in the kidney and appear in the urine, sometimes producing a marked bacilluria. Localisation may also occur in bone-marrow.

The bacillus is found in other lesions occurring as complications or sequelae of typhoid fever—*e.g.* acute suppurative osteitis, abscess of the kidney, acute cholecystitis, broncho-pneumonia, empyema, ulcerative endocarditis. Even in the suppurative lesions it may be present in pure culture.

Infection is by ingestion; from the small intestine the organism passes by the lymphatics to the mesenteric glands, and invades the blood probably *via* the thoracic duct ; the liver, gall bladder, spleen, kidney, bone-marrow, etc., then become infected from the circulation ; from the gall bladder a further invasion of the intestine results and the bacilli may then appear in the faeces in appreciable numbers (*vide* p. 436).

In 2 to 5 per cent. of convalescents, the typhoid bacillus persists in the body, sometimes for an indefinite period. In such "carriers" the bacilli are present in the gall bladder, or in foci in the kidney, and are excreted in the faeces or urine.

The typhoid bacillus cannot exist as a saprophyte outside the body, but it can survive long enough to be transmitted by some vehicle of infection—*e.g.* sewage and polluted water, shell-fish and vegetables contaminated with human excretal matter, house flies, articles of food contaminated by carriers, etc.. Milk may serve as a culture medium for the typhoid bacillus, so that contamination of milk—*e.g.* by carriers engaged in dairy work—is a likely source of outbreaks of the disease.

Serological Reactions.—The serum of animals immunised with typhoid bacilli contains agglutinating antibodies (*vide* p. 40) which are specific for the characteristic antigens of the organism, and an antiserum is therefore employed in the identification of this species (*vide* p. 435).

The *antigenic structure* of this organism in relation to allied species is shown in the Table on p. 450.

Vi antigen and agglutinin.—Recently isolated strains of *B. typhosus* have been shown to possess a somatic antigen (designated " Vi ") which is associated with virulence (judged experimentally by inoculation of mice) and renders the organism relatively inagglutinable by an O antiserum. By continuous cultivation the Vi antigen is apparently lost, the strains become susceptible to O agglutination, and along with these changes there is also loss of virulence.

This antigen is labile at 100° C. and is partially labile to phenol. It seems to be developed best when the organism is cultivated in a soft ascitic-fluid-agar. A Vi agglutinating antiserum is obtained by immunising animals with living typhoid bacilli known to contain the Vi antigen, and absorbing H and O agglutinins from the serum with strains representing H and O antigens but devoid of the Vi component. This absorbed serum can be used in testing strains for Vi antigen. The agglutination is of the granular type. For the detection of Vi agglutinin in serum (*e.g.* in a case of typhoid fever) a selected strain giving a pure Vi reaction may be used, the suspension consisting usually of living organisms. The diagnostic applications of this test are dealt with on p. 442.

Bacteriophage Typing of B. typhosus.—Craigie and Yen have elaborated a method of differentiating Vi strains into types by means of an anti-Vi phage which on serial cultivation with one of these types acquires an increased activity to strains of this type. Thus, phages can be obtained which in certain dilutions act selectively. On this basis eleven types of *B. typhosus* have been recognised. In this way strains can be classified and the method has proved valuable in epidemiological studies of typhoid infections, *e.g.* in correlating cases and in tracing the source of an outbreak and its mode of spread. The technique of the method is described in the following papers: Craigie, J., and Yen, C. H., *Canadian Pub. Health J.*, 1938, **29**, 448 and 484. See also Boyd, J. S. K., *Brit. Med. J.*, 1939, ii, 902.

BACILLUS PARATYPHOSUS *A* (*Salmonella paratyphi*)
BACILLUS PARATYPHOSUS *B* (*Salmonella schottmülleri*)

Causal organisms of paratyphoid fever, which is, for all practical purposes, clinically similar to typhoid.

The morphology and general cultural characters are identical with those of *B. typhosus*.

For their biochemical reactions see Table on p. 470*a*. The chief sugar reactions are: lactose—no change; glucose, mannitol and dulcitol—acid and gas; saccharose—no change. Thus the paratyphoid bacilli can be distinguished biochemically from *B. typhosus*. Biochemical differences between the paratyphoid bacilli may also be elicited (see Table); e.g. *B* ferments xylose and in culture produces hydrogen sulphide whereas *A* lacks these properties. The ultimate identification depends on agglutination reactions.

Their *occurrence* and *distribution* in the body of an infected person are the same as in the case of the typhoid bacillus.

B. paratyphosus A is more common in the East, whereas *B. paratyphosus B* is the commoner in Europe. In recent years in this country *B. paratyphosus B* infections have assumed a greater relative prevalence than formerly. Many of the cases are of a mild type.

Serological Reactions.—These organisms possess different antigens, and agglutinating antisera for known strains are used for the identification of the respective types. The paratyphoid bacilli are similarly differentiated from *B. typhosus.*

The antigenic structure of these organisms in relation to other members of the *Salmonella* group is shown in the Table on p. 450.

Types of B. paratyphosus B

(1) Strains have been classified by Kristensen and Bojlèn into types according to the rate of acid-production in three fermentation tests: (1) fermentation of rhamnose; (2) the so-called Bitter test; and (3) fermentation of inositol.

The rhamnose and inositol media consist of 1 per cent. peptone, 1 per cent. Liebig's meat extract, 0·5 per cent. sodium chloride, and 0·5 per cent. of the fermentable substance. The medium is standardised to pH 7·5–7·6 and the indicator added is bromo-thymol-blue (per litre of medium, 12 c.c. of a solution containing 1 gram to 500 c.c. N/200 NaOH).

The Bitter test consists in growing the organism in a synthetic medium containing rhamnose, methyl red (0·5 per cent. in 96 per cent. alcohol) being added to ascertain whether the pH has been lowered to a certain point. The medium is as follows: 0·5 gram di-sodium hydrogen phosphate, 1·0 gram ammonium sulphate, 2·0 grams sodium citrate, 5·0 grams sodium chloride, 0·05 gram peptone, 1000 c.c. distilled water, with 0·5 per cent. rhamnose added, the pH being about 7.

The rhamnose, inositol and Bitter media are inoculated with a large loopful of an 18 hours' broth culture. The result of the rhamnose test is read after 12 hours' incubation, yellow (indicating acid) being " positive." After 15 hours 2 or 3 drops of methyl-red solution are added to the Bitter culture, a " positive " reaction being indicated by an orange-red to purple colour. The inositol test is read after 18–24 hours, yellow being "positive."

Strains are then classified according to the following system :

	Rhamnose	*Bitter*	*Inositol*
$R_1 l_1$	+	+	+
$R_1 l_2$	+	+	—
$R_2 l_1$	+	—	+
$R_2 l_2$	+	—	—
$R_3 l_1$	—	.	+
$R_3 l_2$	—	.	—

$R_2 l_1$ and $R_3 l_1$ are the commonest types. Such typing of strains may be applied in epidemiological studies, *e.g.* in correlating cases and tracing sources and spread of outbreaks ; but it cannot be claimed that the types are absolutely stable. (See Warren, S. H., and Iredale, J. L. G., *J. Hygiene*, 1934, **34**, 203.)

(2) Bacteriophage typing of *B. paratyphosus B.* has also been used recently for epidemiological studies as in the case of *B. typhosus* (*vide*, p. 433 ; also Felix, A., and Callow, B. R., *Brit. Med. J.*, 1943, ii, 127).

DIAGNOSIS OF ENTERIC INFECTIONS

The bacteriological diagnosis depends on (1) the isolation from the body, and the identification, of the causative organism, or (2) the demonstration of its presence in the body by the Widal agglutination reaction, which is based on the occurrence of specific agglutinins to the organism in the serum of the infected person.

In the early stage of the illness, blood culture is the most conclusive diagnostic method, and should be employed in all cases met with during the first seven to ten days, and in relapses (where a bacteriological diagnosis has not previously been established). The possibility of demonstrating typhoid-paratyphoid bacilli in the blood is much less at a later stage. The method is referred to on p. 182. If the result is positive the strain is isolated in pure culture, and identified by morphological, cultural and biochemical characters (see Table, p. 470*a*), and by testing it with an agglutinating antiserum to a known *B. typhosus*, *B. paratyphosus A*, or *B. paratyphosus B*, according to the biochemical reactions observed. Agglutination should occur approximately to the titre of the serum (for the strain used for immunisation). H and O agglutinating antisera for these organisms can be obtained from the Standards Laboratory, Oxford University. Further reference to their serological identification is made later (p. 448).

The typhoid and paratyphoid bacilli may also be isolated from faeces and urine.

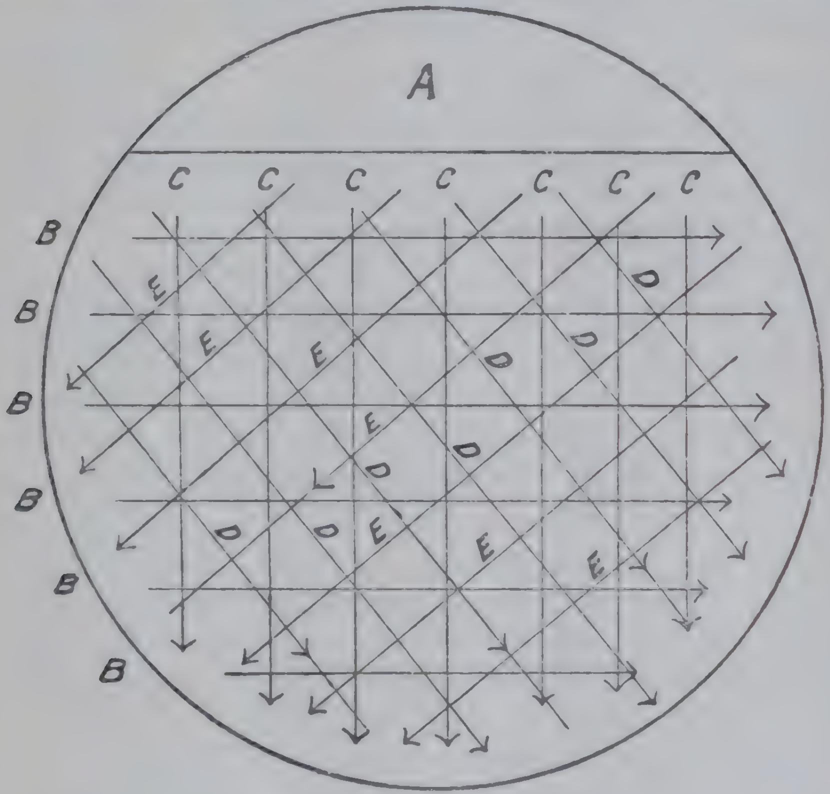
FAECES.—These organisms are most frequent at the end of the second week or during the third week, but may be detected at various stages of the disease. Examination of faeces may frequently yield negative results unless repeated, and the isolation of typhoid-paratyphoid bacilli from this source is often rendered difficult owing to their being relatively scanty as compared with *B. coli*.

In submitting specimens of faeces for culture, if there is likely to be a delay of some hours before the laboratory cultivation can be carried out, 2 volumes of 30 per cent. neutral glycerol in buffered 0·6 per cent. sodium chloride solution (p. 319) should be added to 1 volume of the faeces and thoroughly mixed with it. This obviates the overgrowth of the typhoid-paratyphoid bacilli by the other intestinal organisms present. The glycerol solution is apt to become acid on keeping, in which condition it is quite unsuitable for use. To check this it may be tinted with phenol-red, and if this indicator changes to a yellow colour the fluid should be discarded and a fresh solution used.

Direct plating of Faeces.—A medium is required which will differentiate colonies of typhoid-paratyphoid bacilli from those of the ordinary coliform bacilli; in the past MacConkey's bile-salt neutral-red lactose agar (p. 137) has been extensively used for this purpose, but recently has been generally superseded by desoxycholate-citrate agar which also contains lactose and neutral red (p. 138 and **Appendix**). On both these media *B. typhosus* colonies are "pale" as compared with the pink colonies of *B. coli*. *It is essential that the surface of the medium should be sufficiently* *dry before inoculation.* If even a small amount of condensation water is present on the medium, a confluent growth may result instead of separate colonies.

When *MacConkey's medium* is used the inoculation is made as follows :—

A loopful of the specimen (liquid faeces or a dense emulsion in saline from solid or semi-solid faeces) is



smeared over area A of the plate (see diagram). The loop is sterilised in the flame, re-charged by rubbing it over area A, and then used to inoculate the remainder of the plate by successive parallel strokes, B, C, D and E drawn in the directions indicated in the diagram. The wire should be held so that the whole loop is in contact with the surface of the medium. In this way the resulting colonies are evenly distributed over the plate. The result depends on the concentration of organisms in the specimen, and on the size of plate used. A plate of 6-in. diameter may be employed or alternatively the inoculum is distributed, as already described, on two 4-in. plates. It is essential that the medium be abundantly inoculated, but if the specimen contains an excess of coliform organisms, it may be difficult to obtain satisfactory separation of colonies on a small plate. The method described allows a heavy inoculation to be made with the resulting colonies well separated (except of course in area A).

When *desoxycholate citrate agar* is used the procedure is similar to that described above, but one 4-in. plate suffices and a much heavier inoculation can be made as the medium is relatively inhibitory to *B. coli.*

After eighteen to twenty-four hours' incubation the colonies are usually sufficiently large for " picking off " those that are considered likely to belong to the enteric group. The plates may also be incubated for longer periods if no suspicious colonies are noted after twenty-four hours, and occasionally this procedure is of value. The colonies of *B. typhosus* and *B. paratyphosus* can be recognised by their " pale " appearance, but other intestinal organs may produce colourless colonies on MacConkey's medium. Several of these colonies are therefore subcultured on agar slopes, and for this purpose a straight wire is used for subinoculating them from the plate. The pure cultures isolated are tested and identified as indicated under blood culture (*vide supra*).

The Brilliant Green Enrichment Method for the Isolation of the Enteric Group from Faeces.—This method depends on the selective inhibition of the prevalent types of *B. coli* by brilliant green. Thus, in fluid media containing certain concentrations of the dye, the typhoid-paratyphoid bacilli grow well, while the growth of *B. coli* is partially or completely restrained, and the former organisms can be enriched at the expense of the latter. Occasionally, however, unusual types of coliform bacilli which are resistant to brilliant green may overgrow the enteric organisms, and direct plating (*vide supra*) should be carried out in parallel with the enrichment.

Method.—If the stool is solid or semi-solid, a dense suspension is prepared in sterile saline solution, and from this, or the liquid stool, three tubes of 10 c.c. of peptone water (1 per cent. peptone, 0·5 per cent. sodium chloride, pH 7·0), containing the following amounts of a 1:10,000 watery solution of brilliant green, are inoculated :

(1) 0·25 c.c. (2) 0·4 c.c. (3) 0·7 c.c.

The 1:10,000 solution is made up when required from a stock 1 per cent. solution by the addition of 0·1 c.c. to 10 c.c. of sterile water, and the appropriate amounts are added to the peptone tubes.

In general a large loopful of liquid faeces or faecal suspension is added to the tubes. If, however, the stool is watery, three or four loopfuls should be used for the inoculation of each tube. The tubes are incubated at 37° C., and, after twelve to eighteen hours, subcultures on MacConkey plates are made from each.

Under favourable conditions almost pure growths of the enteric organisms may be obtained in one or more of the brilliant green tubes. Even when pure growths do not result, the typhoid or paratyphoid bacilli are relatively numerous as compared with *B. coli*, so that their isolation is more easily accomplished. This method yields even better results with *B. paratyphosus* than with the typhoid bacillus.

The procedure may be abridged by using only concentration (2) of brilliant green.

Tetrathionate Medium for Enrichment of Typhoid-paratyphoid Bacilli.—This method has also been found valuable for isolating typhoid and paratyphoid bacilli from faeces by their selective enrichment, as in the brilliant green method. The medium is prepared as follows : To 90 c.c. of ordinary broth add 2·5 grams of chalk (previously autoclaved at 10 lbs. pressure and then dried) and sterilise the mixture by steaming for half-an-hour. Add to the chalk-broth 10 c.c. of a 60 per cent. solution of crystallised sodium thiosulphate solution (sterilised by steaming for 30 minutes) and 2 c.c. of iodine solution (prepared by grinding in a mortar 6 grams of iodine and 5 grams of potassium iodide and dissolving in 20 c.c. distilled water). Distribute in 5 c.c. amounts in tubes or screw-capped bottles. A tube or bottle of the medium is inoculated with faeces and incubated for 18–24 hours when a sub-inoculation is made on MacConkey's medium.

Wilson and Blair's bismuth-sulphite medium may also be used with advantage to facilitate the isolation of enteric group organisms (*vide* p. 141).

In the examination of faeces from enteric cases the best results are obtained by employing two or three different methods simultaneously. This gives a higher percentage of positive results than when one method only is used. Thus, direct plating on desoxycholate-citrate medium and the brilliant green and tetrathionate enrichment methods may be used together, or direct plating on Wilson and Blair's medium may be substituted for one of these methods.

Urine.—The urine is centrifuged, several loopfuls of the deposit are inoculated on a plate of MacConkey's or desoxycholate-citrate medium, and successive strokes made in the usual way so that isolated colonies are obtained. In enteric fever there appear to be transient bacilluric periods, and repeated examinations of the urine are of particular value where the isolation of the causative organism is aimed at, and where other methods have been unsuccessful.

Examination of bile.—Another method which may be used for bacteriological diagnosis in the later stages of the illness is the aspiration of bile by means of the duodenal tube, cultures being made from the bile on MacConkey's or desoxycholate-citrate medium.

Widal Reaction.—The technique is described in Chapter VIII. It is now customary to test the serum with standard H and O suspensions (in parallel) of each enteric group organism likely to be encountered—*e.g.* in this country *B. typhosus* and *B. paratyphosus B*. As a rule, both O and H agglutinins are developed, but in some cases only one of these agglutinins is demonstrable, particularly at an early stage.

The reaction becomes definitely manifest *usually* about the seventh to the tenth day. Occasionally it is earlier in development (*e.g.* fifth day), but may be delayed. A negative result at an early stage of the illness therefore may be inconclusive. The Widal reaction, tested quantitatively, is also progressive up to a certain point—*i.e.* the titre of the reaction rises from the time of the first appearance of agglutinins in the serum and reaches its maximum about the end of the third week. A " rising titre " is therefore highly significant in diagnosis.

Enteric carriers usually exhibit a positive reaction (*vide infra*).

Persons inoculated with the typhoid-paratyphoid vaccine also show specific agglutinins in their sera, and this complicates the interpretation of the Widal reaction in such persons.

In previously vaccinated cases a definitely rising titre for any one of the enteric group has been regarded as significant from the diagnostic standpoint.

It has been claimed also that in such persons the agglutinins are mainly of the H type, whereas in infected subjects both O and H agglutinins can be demonstrated. Recent work has shown that in such cases a reliable diagnosis cannot be made merely by the demonstration of O agglutinins in the serum, since vaccination may give rise to O agglutinins as well as H, though the former have usually a lower titre and decline more rapidly.

In vaccinated persons contracting *B. typhosus* infection the Vi agglutination reaction is of special value from the diagnostic standpoint as it seems to depend on the presence of the *living* organism in the body ; thus, a positive result would indicate infection. A method for carrying out this reaction is given later (p. 442).

It must be remembered that normal serum may also agglutinate the typhoid and paratyphoid bacilli in low dilutions, and no diagnostic significance can be attached to the Widal reaction unless the titre is beyond the range of such normal reactions. In this country the following are the usual limits of normal agglutination (the figures refer to " standard titres " as obtained by the method of the Standards Laboratory, p. 257) : *B. typhosus*, H 1 in 30, O 1 in 50 ; *B. paratyphosus B*, H 1 in 30, O 1 in 50 ; *B. paratyphosus A*, O and H, 1 in 10. It must be noted, however, that normal agglutination of these organisms may vary in degree in different communities and different countries.

If agglutination occurs only in low dilutions within the possible range of normal agglutination, the test should be repeated. Later results may show higher titres and are therefore more conclusive.

DIAGNOSIS OF TYPHOID AND PARATYPHOID CARRIERS

In a considerable proportion of carriers the Widal reaction is positive, and the test is of some value as a preliminary one. A negative Widal reaction does not of course exclude the carrier state. In detecting carriers of *B. typhosus* the Vi agglutination test is of particular value.

The proof that a person is a carrier depends on the isolation of *B. typhosus* or *B. paratyphosus* from the faeces or urine, and at least three successive examinations should be made before the result is declared negative.

As the bacilli are likely to be most numerous in the bile and in the contents of the small intestine, three grains of calomel followed by a saline purgative should be given, and after catharsis, the second or preferably the third stool is used for the examination. The specimen should be cultured as soon as possible after it is passed. The methods used are as described above. It is essential that at least two different methods should be used together including an enrichment method.

The urine is examined as in the diagnosis of enteric fever.

The Vi Agglutination Test

The bacterial suspension should be prepared from a selected strain which responds only to the Vi agglutinin and is not acted on by the H and O agglutinins. Such suspension in concentrated form can be obtained from the Standards Laboratory, Oxford University. It loses sensitiveness on keeping and should not be used after two months. The test mixtures are made up and incubated in 3 by ½ in. test-tubes. A series of doubling dilutions of the serum is prepared with graduated pipettes (*vide* p. 250), the initial dilution being 1 in 10 and the last tube in the series being 1 in 640. An additional tube is included for control purposes containing saline only. The amount of each dilution should be 1 c.c. One drop (0·05 c.c.) of the suspension is then added to each tube. Incubation is carried out at 37° C. for two hours and the tubes are then allowed to stand at room temperature overnight. To observe the result the tubes are examined in ordinary daylight, being held somewhat tilted, and the type of sediment determined with the aid of a hand-lens. In the control the sedimented organisms should form a small circular well-defined compact deposit. If marked agglutination has occurred the deposit, consisting of clumped organisms, is scattered over the foot of the tube. Intermediate degrees will also be observed. According to the Standards Laboratory, " standard agglutination " is denoted by absence of the central deposit and bacterial clumps occupying about half the area of the foot of the tube. It has been pointed out that sera with haemolysed red cells may give false positive reactions in low dilutions.

In cases of suspected typhoid fever standard agglutination in a titre of 1 in 10 is considered significant, but repeated tests and demonstration of a rising titre would make the result more conclusive. In suspected typhoid carriers a titre of 1 in 10 would also be regarded as suggestive.

Typhoid-paratyphoid (T.A.B.) Vaccine

This is prepared from selected " S " cultures of *B. typhosus*, *B. paratyphosus A* and *B*, according to the method described on p. 284. The vaccine is sterilised at 60° C. (thirty minutes). For prophylactic use two doses are usually given subcutaneously at intervals of seven to ten days.

	1st dose	*2nd dose*
B. typhosus .	500 millions	1000 millions
B. para. A .	250 or 375 millions	500 or 750 millions
B. para. B .	250 or 375 ,,	500 or 750 ,,

The prophylactic value of this vaccine has been fully established.

B. paratyphosus C may also be incorporated in the vaccine which is then designated "T.A.B.C."

It has been emphasised that the full immunising potency of typhoid-paratyphoid vaccine depends on the use of virulent strains containing adequate O and Vi antigen, but if the vaccine is killed by heat and preserved with phenol it will not stimulate the production of Vi antibody. For this reason Felix and his co-workers have recently advocated treatment of the cultures with alcohol in place of heating and phenolisation. A vaccine prepared in this way from strains selected as above mentioned stimulates the formation of Vi antibody in a substantial proportion of cases.

Anti-typhoid Therapeutic Serum

Antisera prepared by immunising with living typhoid bacilli containing the Vi antigen have recently been advocated for the treatment of typhoid infections. Results so far have not been encouraging.

BACILLUS PARATYPHOSUS *C*

This organism has been reported in cases of enteric fever in different parts of the world. It is closely related to *B. suipestifer* though exhibiting certain differences from the latter (see Tables on pp. 450, 470*a*). It can be distinguished serologically from the other paratyphoid bacilli.

Enteric infections due to the monophasic type of *B. suipestifer* (Kunzendorf) have also been observed (see Tables, pp. 450, 470*a*).

ISOLATION OF TYPHOID AND PARATYPHOID BACILLI FROM WATER

Various methods have been used for the purpose, but it is difficult to isolate these organisms even from a water known to be polluted with infective excretal matter. The following method is recommended for the purpose—to 900 c.c. of water in a sterile screw-cap bottle 100 c.c. of a sterile 10 per cent. solution of peptone containing 5 per cent. sodium chloride are added, and then 5 c.c. of a 1:1000 solution of brilliant green in distilled water. The flask is incubated and subinoculations are made on MacConkey plates after twenty-four and forty-eight hours. The enrichment principle applied in this method is referred to on p. 438.

Wilson and Blair's medium (*vide* pp. 141, 439) has been used successfully in isolating *B. typhosus* from water.

THE FOOD-POISONING GROUP (*Salmonella*)

These organisms are associated with cases of "meat or food poisoning," in which the illness usually takes the form of an acute gastro-enteritis with marked toxaemia. Different types of these organisms vary to some extent in their invasiveness. In some cases bacteraemia or septicaemia may occur, and meningitis has been recorded not infrequently as a result of the infection. Cholecystitis may also result. The incubation period may be short, symptoms occurring even a few hours after the ingestion of the contaminated food. Preserved meats (*e.g.* sausage, brawn, etc.) are frequently responsible. The food may not show any obvious evidence of bacterial contamination. The *Salmonella* organism growing in the food produces an active endotoxin which may be responsible for the early symptoms in cases in which the incubation period is short. This toxin is generally stable at 100° C. for thirty minutes.

Occurrence.—These organisms are found in the intestinal contents during the disease, and in some cases in the blood.

As regards the source of the infection : the animal from which the flesh is derived may have been infected during life, or the meat may have been contaminated from some extraneous source. Organisms of this group occur in certain animals—*e.g.* cattle and pigs. Cow's milk and milk products may be a source of infection. *Salmonella* organisms also produce epizootic infections in rats, mice and other rodents and in certain birds. They have been found in the eggs of infected pigeons and ducks, and cases of food-poisoning in the human subject have occurred after the consumption of such infected eggs. Human carriers have been noted, and these may be responsible for contamination of foods.

Recently attention has been drawn to *Salmonella* infections of man derived from dogs and cats.

BACILLUS ENTERITIDIS OF GAERTNER

(*Salmonella enteritidis*)

This organism is similar to *B. paratyphosus B* in its various general characters (*vide* Table, p. 470*a*), but does not ferment inositol. Its serological identification is dealt with on p. 448. It may be noted here that the somatic antigen of *B. typhosus* is the same as that of *B. enteritidis* (see Table on p. 450).

This organism is responsible for infection in cattle and in rats. It is extremely virulent to laboratory animals by parenteral inoculation, and even by enteral administration of culture produces a haemorrhagic enteritis and septicaemia.

BACILLUS AERTRYCKE (*Salmonella aertrycke* or *S. typhi-murium*)

Similar to *B. paratyphosus B* in cultural and biochemical reactions (*vide* Table, p. 470*a*). In serological characters it is also closely related to this organism. Thus *B. paratyphosus B* is agglutinated, often to titre, by *B. aertrycke* antisera, and similarly *B. paratyphosus B* antisera agglutinate *B. aertrycke*. This depends on the fact that the two organisms possess certain antigenic constituents in common, though each in the specific phase contains a different flagellar antigen (see Table on p. 450). Absorption tests, however, differentiate these organisms (*vide* p. 261).

This is illustrated as follows :—

	Agglutination of—	
	B. aertrycke	*B. paratyphosus B*
Antiserum for *B. aertrycke*	+	+
After absorption with *B. paratyphosus B*	+	–
After absorption with *B. aertrycke*	–	–

For purposes of diagnostic identification, an unknown strain should be able to absorb from a *B. aertrycke* antiserum the specific agglutinins for this organism.

The identification of *B. aertrycke* and other *Salmonella* types is now generally carried out by the method of antigenic analysis which is dealt with in further detail later (p. 448). In this method the absorption technique is used to confirm the results of direct agglutination.

B. aertrycke produces epizootic enteritis in guinea-pigs and mice ("mouse typhoid"). It is less frequently found in rats. Infection occurs in pigs and in certain birds, *e.g.* ducks (*vide supra*).

OTHER TYPES OF THE SALMONELLA GROUP FOUND IN FOOD POISONING

B. aertrycke is the commonest cause of food poisoning in this country ; *B. enteritidis* is second in prevalence. Other types, designated "Thompson" and "Newport," are not infrequent in occurrence. Less common types are *B. suipestifer* and the so-called "Stanley," "Derby," "Potsdam," "Senftenberg var. Newcastle" and "Eastbourne" types, and a considerable number of varieties of the *Salmonella* group has now been described in this country and other parts of the world (see Table on p. 450). These organisms exhibit the general biological characters of the group and are differentiated by their biochemical and serological reactions.

B. suipestifer (*Salmonella cholerae-suis*—Bacillus of hog-cholera) was originally regarded as the causal agent of swine fever or hog-cholera, which is now considered to be due to a filterable virus. The biochemical reactions of this organism are given in the Table on p. 470*a*, and reference to it has been made on p. 444 in relation to "*B. paratyphosus C*." Identification is established by the method of antigenic analysis described on p. 448 (see also Table on p. 450). Two forms of this organism are met with : a diphasic type, and a variety designated "Kunzendorf" which is monophasic, occurring only in the group or non-specific phase.

DIAGNOSIS

The stools are plated on desoxycholate-citrate medium as in the diagnosis of enteric fever, "pale" colonies are picked off, and the resulting cultures are tested and identified. It is advisable also to employ selective enrichment, *e.g.* the brilliant green method (p. 438). Detailed agglutination and absorption tests are necessary for identification of the *Salmonella* type present (*vide infra*).

Blood culture may in some cases yield positive results, and should be carried out as a routine measure. The culture is made by the same method as in enteric fever.

In convalescence, the serum of patients agglutinates the homologous organism, but the agglutination test is not applicable during the acute stage, as the agglutinins take some days to make their appearance in the blood.

The test may sometimes be applied retrospectively in convalescent cases (previously undiagnosed) for purposes of epidemiological investigation. The serum is tested with specific H suspensions of *B. enteritidis*, *B. aertrycke*, *B. paratyphosus C*, Newport and Stanley types (for the last *B. typhosus* H suspension may be used—*vide* Table, p. 450). The suspensions can be obtained from the Standards Laboratory. If a reaction occurs with one of these in dilutions above 1 in 50 this may be regarded as significant. These suspensions, however, only represent the commoner members of the *Salmonella* group. A suspension consisting of mixed monophasic non-specific *B. aertrycke* and *suipestifer* types can also be obtained from the Standards Laboratory for such retrospective tests, but a reaction with it will merely indicate *Salmonella* infection without any determination of the type. A reaction with this suspension in a titre of over 1 in 50 should be regarded as significant. Of course previous typhoid-paratyphoid vaccination invalidates these diagnostic results.

Bacteriological examination of suspected articles of food, if available, should be carried out.

IDENTIFICATION OF TYPES OF THE SALMONELLA GROUP

When a non-lactose-fermenting bacillus presenting the general characters of this group has been isolated from a case, its precise identification is often a matter of considerable difficulty. The presence or absence of motility should be carefully noted and a complete set of sugar media inoculated (including xylose, arabinose, inositol and rhamnose) along with lead acetate agar. The test for utilisation of d-tartrate should also be included (*vide* Table, p. 470*a*). The biochemical reactions are of value when considered in association with the antigenic structure.

As will be seen from the Table on p. 450, the identification of the O antigen (*vide* p. 41) provides a convenient method of placing any member of the group in one of a number of subgroups. To assign the unknown bacillus to its subgroup, sera prepared by immunising animals with alcoholised suspensions of representative members of each subgroup are used in agglutination reactions with an alcohol-treated (O) suspension of the unknown. A convenient set of such O sera are those for *B. paratyphosus A*, *B. paratyphosus B*, *B. suipestifer* (monophasic), Newport type and *B. enteritidis* Gaertner. Small-flake agglutination to the titre of the serum would indicate the nature of the O antigen present. The results of the direct agglutination reaction may then be confirmed by an agglutinin-absorption test.

The next step is to determine whether the culture (if diphasic) is in the specific or group phase (*vide* p. 41). An H-agglutinating serum prepared against *B. suipestifer* (monophasic) is a convenient reagent for this purpose, its H agglutinin being exclusively of "group" character. If a formolised suspension of the unknown bacillus is agglutinated in large floccules to any extent by this serum, it is in the group phase, and an effort must be made to secure a specific-phase subculture of it before further identification is attempted.

Plates are inoculated so as to yield discrete colonies, and a number are examined. Separate colonies are touched with an inoculating wire and some of the growth in each case emulsified in a drop of a low dilution of *suipestifer* (monophasic) serum on a slide. If the serum is known to give rapidly visible flocculent agglutination with the group antigens this procedure is sufficiently reliable as a preliminary test. A colony, organisms from which do not agglutinate with the group serum, is subcultured as it is probably in the specific phase; it is then retested macroscopically. (The group→specific dissociation of the H antigen may be effected by repeated growth in broth containing any "group" serum diluted 1 : 10.)

A most convenient method for obtaining the specific form of a *Salmonella* organism is as follows: 5 c.c. amounts of nutrient agar containing 0·75 per cent. agar are placed in stoppered 6 × ½ in. test-tubes with a small inner tube open at both ends and with the upper end projecting well above the agar; the medium is sterilised, cooled to 50° C. and then 0·5 c.c. and 1 c.c. of a 1 in 5 dilution of the group-serum

SOME REPRESENTATIVES OF THE SALMONELLA GROUP[1]

Arranged in Subgroups with common O antigens

Designation of organism and associated disease	*O antigen*	*H antigen*		
		Specific phase		*Non-specific phase*
		α-phase	*β-phase*	
paratyphosus A—Enteric fever . .	(I), II, XII	a	—	—
paratyphosus B—Enteric fever . .	(I), IV, (V), XII	b	—	1, 2, . .
aertrycke (*S. typhi-murium*) Food poisoning (gastro-enteritis)	(I), IV, (V), XII	i	—	1, 2, . . .
Stanley	IV, V, XII	d	—	1, 2, . .
Reading	IV, XII	e, h	—	1, 5, . .
Derby	(I), IV, XII	f, g	—	—
abortus-equi—Equine abortion . .	IV, XII	?	e, n, x	—
abortus-ovis—Abortion in sheep . .	IV, XII	c	—	1, 6, . .
paratyphosus C (Hirschfeld)—Enteric fever	VI, VII, (Vi)	c	—	1, 5, . .
typhi-suis—infection of pigs . . .	VI, VII	c	—	1, 5, . .
suipestifer (*S. cholerae-suis*) Food poisoning (gastro-enteritis). Kunzendorf variety of *B. suipestifer* also reported in enteric fever.	VI, VII	c	—	1, 5, . .
suipestifer (monophasic) (*S. cholerae-suis* var. Kunzendorf)	VI, VII	—	—	1, 3, 4
Thompson	VI, VII	k	—	1, 5, . .
Potsdam	VI, VII	l, v	e, n, z_{15}	—
Newport	VI, VIII	e, h	—	1, 2, . .
morbificans bovis—Food poisoning (gastro-enteritis)	VI, VIII	r	—	1, 5, . .
typhosus—Enteric fever	IX, (Vi), XII	d	?	—
enteritidis Food poisoning (gastro-enteritis). Eastbourne and Dublin types also reported in enteric fever.	(I), IX, XII	g, m	—	—
,, var. Dublin	I, IX, XII	g, p	—	—
Eastbourne	(I), IX, XII	e, h	—	1, 5. . .
pullorum—non-motile—Bacillary white diarrhoea of chicks.	IX, XII	—	—	—
gallinarum— ,, —Fowl typhoid	IX, XII	—	—	—
London—Relationships to disease not fully defined	III, X, XXVI	l, v	—	1, 6, . .
Bact. anatum—Infection of ducklings and chicks	III, X, XXVI	e, h	—	1, 6, .
Senftenberg var. Newcastle—Food poisoning (gastro-enteritis) . . .	I, III, XIX	g, s, t	—	—
Aberdeen—Gastro-enteritis . . .	XI	i	—	1, 2, .
Poona—Gastro-enteritis . . .	XIII, XXII	z	—	1, 6,

( ) This antigenic component may sometimes be absent.
. . . The full antigenic formula is not given.

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[1] See classification of the Salmonella Sub-committee of the International Society Microbiology, 1934, *J. Hyg.*, **34**. 333; Kauffmann, F., ***Ztschr. f. Hygiene***, 1937, **120**, 17 and Bornstein, S., *J. Immun.*, 1943, **46**, 439.

(filtered to ensure sterility) are added, giving final concentrations of 1 in 50 and 1 in 25 respectively. The medium is allowed to solidify in the upright position. The agar *inside* the inner tube is inoculated by the stab method with the group-phase culture. The specific forms can then be separated by incubating and subcultivating from the agar *outside* the inner tube. The best results are obtained by employing the shortest period of incubation, *e.g.* from early forenoon to evening or from late evening to early morning. (See Tulloch, W. J., *J. Hygiene*, 1939, **39**, 324.)

When the specific phase has been obtained formolised suspensions are used in agglutination reactions with pure specific-phase H sera prepared against the *Salmonella* types in the subgroup to which the already determined O antigen belongs. Again agglutinin-absorption tests should be used to confirm the results of direct agglutination reactions. (It should be noted that some types exhibit two specific phases, designated *α* and *β*, the H components of these being different.)

In most cases the evidence thus obtained, in association with the biochemical reactions (Table, p. 470*a*), will serve to identify the unknown organism if it is one of the commoner types. In the case of diphasic types the group phase may be investigated in the same way by agglutination and agglutinin-absorption tests with known group-phase antisera. This step will, of course, be necessary in the case of monophasic types found in the group phase only—e.g. *B. suipestifer* (monophasic).

When the strain in question belongs to a less common type with O components other than those of the subgroups referred to above, further tests on the same lines are made with the appropriate antisera. In some cases also it may be necessary to prepare O, H (specific) and H (group) antisera for the strain in question and to carry out reciprocal tests with antigens of various *Salmonella* types.

It should be noted that where the bacillus under investigation has undergone variation to the "rough" form its O antigen can no longer be identified, while a non-motile variant can be allocated only to its appropriate subgroup, since it lacks the H (flagellar) antigens necessary for complete serological identification.

In the Table on p. 450 the numbers and letters designate various antigenic components which have been defined.

The Table illustrates the extreme complexity of antigenic structure and yet close inter-relationship of the organisms of the *Salmonella* group, and also indicates the difficulties, technical and otherwise, of the ultimate identification of an organism which has given rise to an outbreak of food poisoning.

(For further information readers should consult *Bulletin*, Ministry of Health, October 1944, v. 4, p. 177).

BACILLUS PULLORUM (*Salmonella pullorum*)

The causative organism of "white diarrhoea" of chicks.

In general biological characters this organism shows some correspondence to the typhoid-paratyphoid group, but is non-motile. Its biochemical reactions are given in the Table (p. 470*a*); gas formation is not abundant. The organism is present in the faeces, blood and internal organs—*e.g.* liver, spleen—and at autopsy can be isolated readily on nutrient agar. It has also been found in the ovaries of adult birds (which have become carriers), in egg yolks and in the yolk-sacs of developing chicks. The serum of infected birds agglutinates the bacillus, and this reaction has been utilised in controlling the spread of the disease among flocks. Agglutination by a serum dilution of 1 in 50 is diagnostic. An agglutinating antiserum is employed in identifying strains. This organism shows a close serological relationship to *B. typhosus*, possessing the somatic antigen which is also common to *B. enteritidis* (see Table on p. 450).

BACILLUS GALLINARUM (*Salmonella gallinarum—Bacillus of Fowl Typhoid*)

This organism, associated with a disease of fowls characterised by severe anaemia, resembles the typhoid-paratyphoid group in general characters, but is non-motile and does not produce gas from sugars. Its fermentative reactions are shown in the Table on p. 470*a*. It has the same somatic antigen as *B. typhosus* and *B. pullorum*, and is serologically indistinguishable from the latter (see Table on p. 450).

GROUP OF DYSENTERY BACILLI (*Shigella*)

The causative organisms of an acute form of dysentery most prevalent in tropical and subtropical countries, but occurring also in temperate climates. The condition called "asylum dysentery" and many cases of infantile diarrhoea are due to these organisms.

Morphology and Staining.—Non-motile, non-sporing, Gram-negative bacilli about 2–4μ by 0·5μ, but showing a tendency to shorter cocco-bacillary forms.

Culture.—In cultural characters resemble *B. typhosus.* Gelatin is not liquefied.

Biochemical Reactions.—They ferment glucose (without gas production), and in the case of all sugar fermentations are non-gas-producing.[1] The dysentery group can be subdivided into types according to the fermentation of lactose, dulcitol, saccharose and mannitol, the production of indole from peptone and agglutination reactions with specific antisera. The dysentery bacilli first described were those which have been designated *B. dysenteriae* Shiga (*Shigella dysenteriae*), *B. dysenteriae* Flexner and *B. dysenteriae* Y (Hiss and Russell). The Flexner and Y organisms are representative of a fairly-well defined subgroup which may be designated *B. dysenteriae* Flexner (*Shigella paradysenteriae*).

The biochemical reactions are shown in the Table on p. 470*a*. It will be noted that the Flexner strains differ from the Shiga type in their fermentation of mannitol.

The Shiga type is also identified by agglutination with an antiserum to a known Shiga strain, and is clearly differentiated in serological characters from the other types of dysentery bacilli.

Organisms with the biological characters of the Flexner subgroup possess a common antigenic constituent, but can be differentiated into six types each containing a distinctive specific antigen (Boyd). These are at present designated V, W, Z (after Andrewes who described them under such symbols) and "103," "P119" and "88" (type-designations used by Boyd). "88" is serologically similar to an organism which has been called the "Newcastle" dysentery bacillus (*vide infra*) but has the biochemical characters of the classical Flexner type with the exception that it may slowly ferment dulcitol.

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[1] The "Newcastle" dysentery bacillus (*vide infra*) is an exception to this rule.

It has been shown that, by variation, the type-specific antigen may be lost, the group antigen remaining; and probably the Y organism of Hiss and Russell is of this nature. It has long been recognised as possessing serological characters common to the whole Flexner subgroup and in the past a Y agglutinating antiserum has proved a useful reagent for identifying this subgroup. It may be noted that an X type was described by Andrewes but it seems doubtful from recent work whether it is a distinctive type. The "Newcastle" bacillus (*vide infra*) which is serologically related to Boyd's "88" has been included by some writers in the Flexner subgroup but presents distinct differences in biochemical reactions from this subgroup (*vide infra*).

Experimental Inoculation.—Cultures of dysentery bacilli are generally non-pathogenic when introduced by the mouth in laboratory animals. Intravenous injection produces a haemorrhagic enteritis, and, if the animal survives, muscular paralysis may result. These effects are specially marked in the case of the Shiga type, which forms a potent diffusible toxin.

Besides these types, other dysentery bacilli are met with which do not conform to the Shiga or Flexner organisms and yet have the general characters of the dysentery group (as stated above). These have sometimes been designated *atypical dysentery bacilli*, and their biochemical characters are indicated collectively in the Table. Some of them have been clearly proved to be pathogenic; the pathological relationships of others have been questioned. Strains are also met with possessing all the biological characters of the Flexner subgroup but serologically quite distinct from the recognised forms of this subgroup. Three distinct serological types with these characters have been described by Boyd—designated 170, P288 and D1.

He suggests grouping them together as "*B. dysenteriae* India."

Sonne type.—This organism is a frequent causal agent of dysentery in this country. It represents a distinct serological type. In biochemical reactions it resembles the Flexner subgroup, but produces late fermentation of saccharose and frequently lactose. Indole production is, however, absent. The colonies of this organism on a neutral-red lactose medium, *e.g.* MacConkey's or desoxycholate-citrate agar, are at first "pale" and similar to those of other dysentery bacilli. In some cases red papillae develop on the colonies after several days' incubation, and the late fermentation might appear to be a function of biological variation (*cf. B. coli mutabilis*).

Schmitz type.—Similar to *B. dysenteriae* Shiga, but differs in producing indole.

Bacillus alkalescens.—Differs biochemically from the Flexner subgroup in its fermentation of dulcitol. It produces indole and alkalinises milk.

Bacillus dispar.—This type ferments lactose and saccharose (and sometimes dulcitol) as well as glucose and mannitol. It also forms indole and coagulates milk. There has been some doubt whether *B. dispar* is a dysentery-producing organism.

It is difficult to separate the lactose-fermenting types of the "atypical dysentery bacilli" from non-motile organisms classified as *B. coli anaerogenes*, though the latter may occur quite independently of any pathological condition.

"Newcastle" dysentery bacillus.—This designation was originally applied to an organism which fermented glucose, dulcitol and maltose, but had usually no action on lactose, saccharose or mannitol. Under certain conditions it appeared to be non-gas-producing but in a Lemco broth medium gas was formed from the carbohydrates it fermented. Strains of this type are serologically uniform. (Late fermentation of mannitol by some strains has been described.)

As stated above Boyd's type "88" of the Flexner subgroup is serologically similar to this organism.

In our experience strains which may be classified as the "Newcastle" dysentery bacillus ferment glucose but not lactose, saccharose or mannitol. Most strains ferment dulcitol slowly. Usually a small amount of gas is produced from the fermented carbohydrates even in the ordinary peptone water medium. Indole is not usually formed.

The present classification of the dysentery bacilli may be summarised as follows :—

Classical or Typical.—Shiga type—non-mannitol-fermenter. *Flexner subgroup*—mannitol fermenters—6 distinctive serological types, V, W, Z, " 88," " 103" and "P119" (Boyd), " 88" being serologically similar to the " Newcastle" dysentery bacillus (*vide infra*).

Other dysentery bacilli.—" *B. dysenteriae* India " (Boyd)—like Flexner subgroup but serologically quite distinct—3 distinctive serological types, " 170," " P288 " and " D1 " (Boyd).

Sonne type—like Flexner subgroup, but ferments (slowly) saccharose and frequently lactose ; serologically distinctive.

Schmitz type—like Shiga type, but produces indole : serologically distinctive.

B. alkalescens—like Flexner subgroup, but ferments dulcitol.

B. dispar—like Flexner subgroup, but ferments lactose and saccharose.

The " *Newcastle* " *type* differs from other dysentery bacilli in its gas production from fermented carbohydrates, glucose and in some cases dulcitol and mannitol. It is serologically related to Flexner subgroup.

Occurrence.—The dysentery bacilli are found in large numbers in the stools at an early stage of the illness, even in practically pure culture, but become progressively less numerous, until, at a later stage, they are apparently absent. They do not invade the blood stream as a rule. The Shiga type is associated mostly with the acute and severe forms of dysentery, many of the atypical bacilli with the milder cases, and the Flexner type occupies an intermediate position as regards the severity of illness with which it is associated. It may be noted here that the Shiga type differs from the other varieties in its production of a powerful diffusible toxin which, like the exotoxins, is antitoxinogenic. The Schmitz type, however, has been shown to produce a somewhat similar exotoxin.

Concomitants.—When dysentery bacilli tend to disappear from the stool they are found along with, or become replaced by, certain other unusual organisms which may be designated " concomitants."

The commonest are :

Morgan's bacillus (*Proteus morgani*) and allied types	Gram-negative, non-sporing, gelatin-non-liquefying, aerobic bacilli.
Paracolon bacilli	
B. faecalis alkaligenes (*Alcaligenes faecalis*)	

Their biochemical reactions are shown in the Table on p. 470*a*. They are all non-lactose-fermenters and produce " pale " or colourless colonies on neutral-red lactose media. They resemble in this respect *B. typhosus*, the *Salmonella* group and the dysentery bacilli.

Though Morgan's bacillus does not liquefy gelatin it is now classified with *B. proteus*. Thus on 1 per cent. agar at 25° C. it shows the spreading character of the growth of *B. proteus* and it is also serologically related to some strains of this organism.

B. faecalis alkaligenes colonies on MacConkey's medium may be relatively large, and around each colony may be noted a broad light-yellow coloured zone due to the alteration of the neutral red by alkali production.

While strains corresponding in general biological characters to this organism have been described with peritrichous flagella, similar organisms are frequently met with which have terminal flagella like a vibrio and show curved vibrionic forms. Many strains produce a brown growth on potato like the *B. melitensis-abortus* group, and *B. faecalis alkaligenes* resembles these organisms in its lack of fermentative properties. The biological relationships of this organism are still a matter of doubt.

These organisms are also found in cases of non-dysenteric diarrhoea.

Cases of *B. faecalis alkaligenes* bacteraemia have been recorded.

Morgan's bacillus and allied types are considered to be responsible for some cases of infantile diarrhoea in temperate climates.

The *Diagnosis of bacillary dysentery* is dealt with later (p. 463).

Therapeutic antisera for the dysentery bacilli.—These sera are usually polyvalent and are prepared by immunising horses with the main types of dysentery bacilli. They possess antibacterial properties (*vide* p. 36), but are also antitoxic towards the toxin of the Shiga type. The best therapeutic results are obtained in Shiga-type infections. Large doses should be administered, *e.g.* 50–100 c.c., and in severe cases by intravenous injection.

Bacteriophages for dysentery bacilli (*vide* p. 642) have been advocated for the treatment of bacillary dysentery, but their therapeutic value is doubtful.

Chemotherapy.—Considerable success has been obtained in bacillary dysentery from the use of certain sulphonamide compounds, *e.g.* sulphaguanidine.

Bacillus pyosepticus equi (*Shigella viscosa*).—This organism causes purulent nephritis in horses and some cases of "joint ill" in foals. It has also been described as a rare cause of pyaemia in young pigs. It has been regarded as allied to the dysentery bacilli, though its true relationships may be with the pneumobacillus group. It is non-motile and Gram-negative. In the tissues the organisms are found in masses with capsular material around them. Growth is favoured by the presence of serum, but the organism will grow on ordinary media. On agar it produces two types of colonies: mucoid and non-mucoid. In broth it produces a slimy sediment and a viscous supernatant fluid. Glucose, lactose, saccharose and mannitol are fermented (without gas); indole is not produced; gelatin is not liquefied.

THE INTESTINAL PROTOZOA

ENTAMOEBA HISTOLYTICA

The causative organism of one form of tropical dysentery.

Biological characters.—The vegetative forms are large, rounded, elongated or irregular amoebae, varying in diameter from 10–50μ, the average size being about 20–25μ. The cytoplasm consists of a clear hyaline ectoplasm, and a granular, often vacuolated endoplasm, but this differentiation is not always readily observed. In their most active condition the amoebae show flowing movements, but in a less active state they rapidly protrude and retract pseudopodia, which may be composed mostly of ectoplasm. The movements are mainly changes in shape. The nucleus is round or oval, and in the unstained condition is not easily distinguished. It is situated in the endoplasm, usually excentric in position. It is poor in chromatin, and the nuclear membrane is thin. The chromatin granules are small, and are collected in a ring just inside the nuclear membrane. The nucleus shows a small central karyosome. The amoebae ingest red corpuscles, leucocytes and tissue cells, which are observed in the endoplasm, but ingested bacteria are rarely found. The ingested erythrocytes appear smaller than normal. The vegetative forms after leaving the body tend to become rounded and immobile, and soon die and disintegrate. Multiplication is by amitotic binary fission.

Under conditions unfavourable to the amoebae—*e.g.* when the disease is becoming arrested—encystment occurs. Cysts are more or less spherical, with a thin hyaline refractile cyst wall, which gives them a distinct double contour. The contents are finely granular. The average diameter is 9–14μ. The cysts usually contain multiple nuclei, *not more than four*, a glycogen mass, and also thick rod-shaped or oval structures which stain deeply with haematoxylin and are called " chromatoid bodies."

The cysts are developed by division of the vegetative form into smaller and rounded " precystic " forms.

The newly formed cyst has only one nucleus which later divides into two with further division to four. The glycogen mass is best seen in young cysts, staining brown with iodine, but is apparently used up as the cyst matures. In unstained preparations the chromatoid bodies appear as refractile structures.

Methods of microscopic demonstration and staining are referred to under dysentery diagnosis.

Occurrence.—In the early stage of amoebic dysentery the vegetative forms are present in considerable numbers in the large intestine and in the stools. They penetrate the mucosa of the large bowel and disintegrate the tissue by their pseudopodia and probably also by means of a liquefying ferment. The submucosa is invaded, and, occasionally, small veins are penetrated from which the amoebae may be carried to the liver. In the bowel, oval or irregular ulcers are developed with undermined edges, which may sometimes lead to perforation of the bowel wall. There is little inflammatory reaction (*cf.* bacillary dysentery) unless a secondary septic infection occurs.

Cysts may be detected in the stools, often in large numbers in chronic cases. After apparent recovery the patient may remain a carrier, and the encysted forms are passed in the faeces. The cyst represents a resting phase with increased powers of resistance and can survive outside the body for considerable periods. This is the form in which the organism is transmitted from person to person.

Cyst carriers may also be found who have no history of previous dysentery but it seems likely that such persons have small lesions of the bowel insufficient to produce obvious clinical signs of the disease.

The so-called " tropical abscess " results from invasion of the liver probably through the portal circulation. The abscess contains a slimy chocolate-coloured " pus " consisting of necrotic tissue and altered blood, with only a few leucocytes or pus cells. The amoebae are found mainly in the wall of the abscess, and may not be present in the pus when first evacuated.

Cultivation.—This organism can be cultivated artificially by the method of Boeck and Drbohlav (p. 154).

Experimental Inoculation.—Infection can be produced in kittens by feeding them with material containing cysts: a condition resembling the human disease results. It has been stated that rats can also be infected.

ENTAMOEBA COLI

A non-pathogenic intestinal amoeba which in diagnosis must be carefully differentiated from *E. histolytica.*

The vegetative forms closely resemble those of *E. histolytica*, but the cytoplasm is not so distinctly differentiated into endo- and ecto-plasm. The pseudopodia are small and delicate and not so refractile as those of *E. histolytica.* The nucleus is usually central in position, easily distinguishable, rich in chromatin which is sometimes arranged in quadrant form, and has a thick refractile nuclear membrane. The karyosome is well marked. Amoeboid movement is sluggish. It has been generally agreed that no ingested red cells are seen in the cytoplasm (when this organism is noted in a case of dysentery). It has been pointed out, however, that *in vitro* this organism can ingest red cells as readily as *E. histolytica* (Dobell). Bacteria are ingested often in large numbers. The cysts are larger (15–20μ) than those of *E. histolytica*, the cyst wall is thick, and there are more than four nuclei—*e.g.* frequently eight. No bar-shaped chromatoid bodies are observed in the fully developed cysts which occur in the faeces.

Endolimax nana.—A frequent non-pathogenic intestinal amoeba. The vegetative form is 9μ in diameter or less. In unstained preparations the nucleus is not distinct, but when stained by haematoxylin it is easily demonstrated, and shows a large irregular excentric karyosome.

The cysts are oval, and 7–9μ in their longest diameter. They contain one, two or four small nuclei, but no chromatoid bodies.

Iodamoeba bütschlii and Dientamoeba fragilis are also included among the intestinal amoebae of man, but need not be described here. Their characters may be ascertained by reference to works on protozoology.[1]

Entamoeba dispar.—This designation has been given to an entamoeba which closely resembles small forms of *E. histolytica.*

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[1] See *Protozoology*, Wenyon, London, 1926.

Thus the encysted forms may be quadrinucleate. It is regarded as non-pathogenic. There is some doubt whether it constitutes a species distinct from *E. histolytica* or is a non-virulent form of this organism.

INTESTINAL FLAGELLATES

These organisms are associated with dysentery and diarrhoea particularly in the tropics, but their pathogenicity is doubtful, and they may occur as commensals.

Trichomonas hominis.—Is pear-shaped, 9–15μ long, and shows a nucleus and cytostome. It possesses three or four flagella projecting from the broad end, and also another flagellum forming the border of an undulating membrane and with the free part projecting from the pointed posterior end.

An organism which is biologically similar to *Trichomonas hominis* may occur in the vagina and has been named *Trichomonas vaginalis.* It may be found in cases of vaginitis and though regarded by some as pathogenic its aetiological relationships have not been fully established. For its recognition " wet " preparations of vaginal secretion should be examined first with the lower power of the microscope and then with a $\frac{1}{6}$-in. lens. Dried films stained by Leishman's stain may also be used for diagnostic examination. Its morphological features are similar to those of *T. hominis.*

Chilomastix mesnili.—Resembles *T. hominis*, but has no undulating membrane and only three flagella. It has an elongated slit-like cytostome. Cysts can easily be recognised ; they are oval, about 8μ in their long diameter, and contain one nucleus.

Giardia (*or Lamblia*) *intestinalis.*—Inhabits the duodenum and jejunum.

Main characters :—somewhat flattened in shape ; flat surface pear-shaped ; bilaterally symmetrical ; 10–18μ in its long diameter ; a large sucking disk on one surface ; two nuclei with karyosomes ; two long median parallel axostyles which represent skeletal structures, with blepharoplasts at each end ; eight flagella in pairs—two arising from the anterior blepharoplasts (the broad end is spoken of as anterior), two arising near the anterior blepharoplasts but following the axostyles to the posterior edge of the sucker before diverging, two arising at the posterior edge of the sucker and rooted in the axostyles, and two arising from the posterior blepharoplasts.

The cysts are characteristic : oval in shape, about 10–15μ long, with two or four nuclei (the cyst containing two organisms formed by subdivision) ; the parallel axostyles are observable.

For further information regarding *other intestinal protozoa*, reference should be made to works on protozoology.

DIAGNOSIS OF DYSENTERY

Collections of specimens of stools.—The stool should be examined as soon as possible after being passed, and should be unmixed with urine. The specimen for examination is collected in a faeces specimen tube provided with a cork carrying a metal spoon or scoop which fits into the tube, and by means of which faecal matter may be collected. A universal container may also be used (see p. 318). If the stool contains both faecal matter and mucus, a portion of the latter should be included in the specimen.

When considerable numbers of cases have to be examined for dysentery bacilli specimens may be conveniently obtained by rectal swabs (p. 321). This procedure is specially suitable when examining children.

Microscopic Examination.—A microscope slide is gently warmed over the Bunsen flame, and on one half of the slide a large drop of normal saline solution is placed and on the other a drop of Lugol's iodine (p. 200). A loopful of the stool or the mucous discharge is emulsified in the saline drop and another loopful in the iodine solution. The preparations are covered with No. 1 cover-slips, and examined first with the $\frac{1}{6}$-in. and then with the oil-immersion lens. It is advisable to use a "warm-stage" attached to the microscope in examining fresh preparations for amoebae (*vide* p. 69).

Vegetative amoebae can usually be recognised without difficulty. In the saline preparation *Entamoeba histolytica* may often be identified by its active amoeboid movement and the inclusion in the cytoplasm of numerous red corpuscles (*vide supra*). On the other hand, immobile vegetative amoebae without ingested corpuscles present considerable difficulty in their identification. The presence of cysts facilitates diagnosis owing to the more striking differences between the encysted *E. histolytica* and *E. coli* (*vide supra*). In the iodine preparation the nuclei of the cysts are distinctly seen.

Large phagocytic cells (macrophages) may be found in dysenteric stools, and may be mistaken for immobile amoebae by inexperienced workers. They often show vacuolation, and may even contain red corpuscles. They are immobile, and the nucleus, unless degenerate, occupying one-fourth or one-fifth of the whole cell, is definitely larger than that of an amoeba, and is not of the ring-like or vesicular type. This distinction is seen in the iodine preparation. In a heat-fixed film these macrophage cells and their nuclei can be stained with methylene blue, while amoebae cannot thus be demonstrated.

Other intestinal protozoa that may be present can also be detected in unstained preparations (*vide supra*).

Where pathogenic amoebae cannot be detected, the microscopic examination often yields information of diagnostic importance. In a case of bacillary infection there is usually an abundant and characteristic cellular exudate. The cells present are mostly polymorph leucocytes with a varying number of red cells, and in the early stages, numerous epithelial cells. In addition to these, macrophages are frequently a characteristic feature of the exudate. The pus cells as a rule show marked degeneration.

In amoebic dysentery there are few leucocytes unless the case is also complicated by a bacterial infection. Any leucocytes present are not so degenerate as in bacillary dysentery. Charcot-Leyden crystals are frequently seen microscopically in amoebic dysentery and are absent in bacillary dysentery.

The microscopic examination is therefore an important step in diagnosis: the finding of the characteristic *E. histolytica* establishes a diagnosis of amoebic dysentery, while an abundant cell exudate and the absence of amoebae would indicate bacillary dysentery.

This preliminary determination enables a report to be made at once as to the nature of the dysentery, so that treatment can be initiated without delay.

Where no amoebae can be found, and if a diagnosis of bacillary dysentery cannot be established, it is essential that further microscopic examinations be carried out before amoebic infection is excluded.

Stained preparations are of assistance in the identification of intestinal amoebae. Films are made on cover-slips from the stool and are fixed " wet " by floating the cover-slips (film downwards) in a fixing solution consisting of 2 parts saturated perchloride of mercury in saline, with 1 part absolute alcohol. They are then stained with iron haematoxylin (p. 224).

Culture.—When the clinical and microscopic data point to a bacillary infection, cultures are made from the stool on desoxycholate-citrate medium as in the direct culture of enteric specimens (*vide* p. 436). Until recently MacConkey's medium was extensively used, but has been generally replaced by the desoxycholate-citrate medium which facilitates the detection of dysentery bacilli owing to its inhibitory effect on coliform organisms. In general, however, if cases are examined within the first two or three days the infecting organism can be isolated without difficulty, even on a medium such as MacConkey's which does not inhibit coliform organisms. At a later stage the dysentery bacilli become less numerous and " concomitants " (*vide supra*) are present often in large numbers.

For the complete bacteriological investigation of a case of bacillary dysentery, therefore, it is necessary to consider all the abnormal organisms present in the stool. These are for the most part non-lactose-fermenters, and their colonies, like those of the dysentery group, are of the " pale " type on a neutral-red lactose medium. Certain varieties of dysentery bacilli (*e.g.* the Sonne type) may ferment lactose in fluid medium, but in primary culture from stools present " pale " colonies after twenty-four hours.

Subcultures on agar slopes should be made from each type of pale colony; and since colonies which appear to be similar may represent different organisms, at least three pale colonies should be picked off.

Sufficient growth is usually obtained after twelve hours' incubation to proceed with the examination of the agar slope cultures. Tubes of the following media are inoculated :—

(1) peptone water
(2) glucose peptone water
(3) lactose ,, ,,
(4) saccharose ,, ,,
(5) mannitol ,, ,,
with neutral red or Andrade's indicator, and Durham tube (*vide* p. 121).

and these are incubated for twelve to twenty-four hours. The peptone-water culture is examined after six to seven hours for motility of the organisms.

In this way one can ascertain to what subgroup the various cultures belong. If a culture has the following characters :—

	Motility	*Glucose*	*Lactose*	*Saccharose*	*Mannitol*
Gram-negative bacillus .	–	+	–	–	– or +

—*i.e.* corresponding to the classical dysentery bacilli, *B. dysenteriae* Shiga or Flexner (see Table, p. 470*a*)—the final identification is made by tests with specific agglutinating sera—*i.e.* an antiserum to the Shiga type, and a polyvalent antiserum for the Flexner subgroup. The Standards Laboratory, Oxford University, supplies two polyvalent Flexner sera for this purpose: (I) prepared by immunising animals with V, W, X and Z types, and (II) prepared by immunising with Boyd's types "103" and "P119" and the Newcastle dysentery bacillus (*vide* p. 453).

It should be noted that the Newcastle bacillus after 24 hours usually shows fermentation of glucose only, often with slight gas production. It is identified by means of a monovalent agglutinating serum. It reacts, of course, to the No. II polyvalent serum of the Standards Laboratory. If it is considered necessary for the epidemiological study of outbreaks, monovalent agglutinating sera for each of the Flexner types may be used.

If the unknown strain fails to react to the appropriate antiserum, its biological characters should be thoroughly studied: dulcitol medium should be inoculated, and the peptone-water tube is tested for the presence of indole (see p. 425) after forty-eight hours, and if negative, also after seven to ten days. All fermentation tubes should be incubated for several days before final readings are made. A gelatin stab, or alternatively a coagulated-serum culture, should also be made to test for liquefaction.

If the strain still appears to have the characteristic reactions of a typical dysentery bacillus, the possibility of its belonging to one of the types described by Boyd as *B. dysenteriae* India would have to be considered; their identification would be made by antisera for known stains.

The Sonne and other atypical forms can be identified by their characteristic biochemical reactions and, in the case of the Sonne type, agglutination with a specific antiserum (*vide* p. 455). The Schmitz type can also be identified by means of a specific serum.

Other unusual intestinal organisms (*vide* p. 457) can be identified by completing the cultural tests as in the investigation of the atypical *B. dysenteriae*. Their reactions are shown on the Table on p. 470*a*.

Agglutination Tests with Patient's Serum.—In cases where the causative organism cannot be isolated, agglutination tests have a limited application in diagnosis.

Normal serum may agglutinate the dysentery bacilli in low dilutions (*B. dysenteriae* Shiga in a titre of 1 in 25, *B. dysenteriae* Flexner types in a titre of 1 in 50), and in cases of bacillary dysentery the specific agglutinating effect of the serum towards the actual causative strain isolated from the case may be relatively weak. Normal serum reactions introduce, therefore, a limitation to the diagnostic test. In the case of the Sonne type, however, agglutination in a titre of 1 in 20 is said to be diagnostic.

As in the Widal reaction, the agglutination reaction is only applicable after five to seven days from the onset of the illness.

In general the test is carried out on the same principle as the Widal reaction (*vide* p. 250); known strains of the different types of *B. dysenteriae* are tested in parallel series with varying dilutions of the patient's serum. Standard suspensions of various types are obtainable from the Standards Laboratory, Oxford University.

ENTAMOEBA GINGIVALIS

This organism occurs in considerable numbers in pathological conditions of the mouth—*e.g.* pyorrhoea, gingivitis, dental caries—but has no definite aetiological relationship to these conditions. It is about 10–20μ in diameter and resembles *E. histolytica* in many respects, showing active amoeboid movement and differentiation of the cytoplasm into ecto- and endo-plasm; the nucleus is indistinct in unstained preparations; the organism apparently possesses the property of ingesting free cells—*e.g.* leucocytes.

THE LACTOBACILLI

These organisms constitute a group of acid-resistant (acidophile or aciduric) Gram-positive non-sporing bacilli which occur in the intestine of mammalian animals and are particularly prevalent during the stage of suckling. Thus, in breast-fed infants such organisms may constitute the predominant flora of the intestine, and two main types have been recognised and specially studied.

Organisms of this group are also found in cow's milk, in the human mouth, stomach and vagina, in soil, and in silage and bran.

BACILLUS ACIDOPHILUS (*Lactobacillus acidophilus*)

So called because it is able to flourish in a highly acid medium. It occurs in faeces, saliva and milk. In morphology it is a relatively large, non-sporing, non-motile, Gram-positive bacillus. The individual bacilli vary in length and may appear even in short coccal forms. Some are about 1μ broad, but slender forms may be noted, and there is a tendency to chain formation. The organism thus shows considerable pleomorphism. It may be cultivated readily on whey-agar at 37° C. (*vide* p. 155). The colonies are small, and vary in appearance as seen under the low power of the microscope; two main types are described: (1) "feathery," in appearance not unlike a *B. tetani* colony (*q.v.*) and (2) rounded with projecting outgrowths ("crab-colony"). A convenient method of obtaining cultures from faeces is to inoculate broth to which is added 0·5 per cent. of glacial acetic acid; after incubation subcultures can be made on agar plates under aerobic conditions.

This organism produces acid fermentation of glucose and lactose without gas formation. It also ferments maltose, whereas *B. bulgaricus*, a related organism originally isolated from Yoghurt (a fermented milk), has usually no action on maltose. The latter organism cannot grow in the intestine of man. It is a thermophile, the optimum temperature being 45°–62° C..

Other allied organisms are *B. acidophilus odontolyticus*, described in association with dental caries, the so-called *Boas-Oppler bacillus* found in the stomach contents in conditions in which hydrochloric acid is absent or deficient, and *Döderlein's bacillus* which is found normally in the vagina.

BACILLUS BIFIDUS (*Lactobacillus bifidus*)

Derives its name from the apparently bifid appearance described by the original observers. This organism is found in large number in the faeces of breast-fed infants. Its average dimensions are 4μ by $0{\cdot}5$–$0{\cdot}7\mu$, but it shows considerable pleomorphism. The ends are often expanded. Three bacilli together may be arranged like a Y. Though usually Gram-positive, there is a certain amount of variation in its reaction to Gram's staining method.

In primary culture it is a strict anaerobe. Cultures have been obtained at 37° C. in tubes of neutral lactose-broth containing a piece of sterile rabbit kidney (*vide* p. 150) with a layer of sterile vaseline superimposed on the medium. After several days' growth, subcultures are made on glucose-agar, plates of which are incubated anaerobically. Pure cultures in glucose-agar can be obtained from single colonies. The organism may ultimately become microaerophile. Glucose, saccharose, maltose and various other sugars are fermented with acid production but no gas.

BACTEROIDES

Organisms of this genus (Gram-negative, non-sporing, motile or non-motile anaerobic bacilli) may occur in the intestine of mammals and have sometimes been found associated in the human subject with appendicitis, urinary and puerperal infections, etc.. *Bacillus fragilis* (*Bacteroides fragilis*) is the type-species. It is a small Gram-negative, non-motile, rod-shaped organism sometimes showing bipolar staining and is an obligate anaerobe. Growth can be obtained on ordinary media at 37° C. but is scanty and the colonies are small. Acid (without gas) is produced from glucose, saccharose, maltose and certain other sugars. Gelatin is not liquefied. This species has been found in appendicitis and in suppuration of the urinary system but its pathogenic rôle has not been clearly determined. When injected subcutaneously in guinea-pigs it may produce an abscess, and in rabbits extensive sloughing.

(It may be noted that *B. fragilis* has also been classified by some writers along with *B. fusiformis* (pp. 536, 545) and *B. necrophorus* (p. 536) in one generic group designated *Fusiformis*. The taxonomy and relationships of these organisms require further study.)

CHAPTER XVI

CHOLERA VIBRIO AND ALLIED ORGANISMS; PLAGUE BACILLUS AND PASTEURELLA GROUP

VIBRIO CHOLERAE (*Vibrio comma*)

THE causative organism of Asiatic Cholera.

Morphology.—Curved or "comma-shaped" rods (vibrios) with rounded or slightly pointed ends, about 1·5–3μ by 0·5μ. They are actively motile, and the movement is of a "darting" or "scintillating" type. Motility is due to a single long terminal flagellum. The vibrios occur singly, in pairs, or in chains end to end with the curves alternating—*i.e.* presenting a somewhat spiral arrangement. "S" forms and spirals representing elongated undivided single cells may be noted. Involution occurs readily, especially in culture, and globular, club-shaped or irregular forms may be observed. No spores are produced.

When the organism has been growing in artificial culture for a time, the morphology becomes less typical and the curvature of the vibrios is less pronounced.

Staining.—Gram-negative.

Culture.—Aerobe; slight growth also occurs under anaerobic conditions. Temperature range — 16°–40° C., optimum—37° C.. Grows on ordinary media. A slight trace of acid is inhibitory, but abundant growth occurs on alkaline media—*e.g.* Dieudonné's medium (*q.v.*). The optimum reaction is about pH 8·2.

Colonies on agar—white circular disks about the size of *B. coli* colonies, semi-transparent, with well-defined circular margins; older growths develop a brownish-yellow colour.

Other types of colony may be noted as variants from the standard form—*e.g.* an opaque yellowish-white colony, a "ring" colony with an opaque centre and transparent border, and a "rugose" colony; in the last mentioned, the corrugated growth is due to a gelatinous intercellular substance or a definite capsule (Bruce White).

Gelatin stab—at first there is a white line of growth along the needle track; then liquefaction occurs at the top and spreads downwards in funnel-shaped form.

Coagulated serum is liquefied.

Potato slope—at first, a white layer of growth forms, and later a brownish-yellow or pinkish colour is developed; to obtain this growth the reaction of the potato medium must be alkaline.

In broth—a uniform turbidity results, and a characteristic pellicle forms on the surface.

Biochemical Reactions.—The fermentative reactions are as follows:—

Glucose	Lactose	Dulcitol	Saccharose	Mannitol	Maltose	Mannose	Arabinose
⊥	– (some strains ⊥ after several days' growth)	–	⊥	⊥	⊥	⊥	–

(⊥ =acid; no gas)

They can be tested for as in the case of the coli-typhoid group.

Cholera-red reaction—this depends on the production of indole *and nitrites* in peptone water. It can be elicited by adding a few drops of sulphuric acid to a four-days' peptone-water culture. A reddish-pink colour develops, due to the formation of nitroso-indole.

Haemolysis—the classical type of *V. cholerae* is *non-haemolytic*, but haemolytic vibrios showing a serological relationship to *V. cholerae* may be met with in cholera-like cases and carriers (*e.g.* the "El Tor vibrio").

It is advisable to test for haemolysis by adding varying amounts (0·1–1·0 c.c.) of a two-days' broth culture to 1 c.c. of a 5 per cent. saline suspension of sheep's red blood corpuscles in tubes which are incubated for two hours and allowed to stand overnight in the refrigerator; observations are then made (Greig).

Haemolytic vibrios, such as the El Tor variety, manifest their haemolytic action when growing on blood-agar, but certain vibrios, including strains of *V. cholerae* which do not form haemolysin, also produce clearing or apparent laking when growing on this medium, due probably to a chemical alteration of the haemoglobin. Such vibrios clear coagulated blood media—*e.g.* "chocolate agar"—in the same way as ordinary blood-agar.

Viability.—*V. cholerae* is killed at 56° C. in thirty minutes. It dies within two or three hours when subjected to drying. In stagnant water the organism may survive for a considerable period—*e.g.* eighteen days or longer. The cholera vibrio is readily distributed by water supplies.

Serological Reactions.—For practical purposes the cholera vibrio may be regarded as a homogeneous species, and unknown strains can be identified by testing their agglutination reaction with an antiserum for a known *V. cholerae.*

See **Appendix**—serological types of *V. cholerae.*

The organism possesses both H and O antigens. The H antigen may be shared with certain other vibrios—*e.g.* paracholera vibrios (*vide infra*)—though these organisms are distinct as regards their O antigens. This serological difference is best demonstrated with bacterial suspensions made up in plain saline solution (without formalin). Most of the El Tor strains (*vide supra*) possess the same H and O antigens as the classical cholera vibrio.

Transformation of a *V. cholerae* strain to the "rough" form is associated with loss of the specific O antigen.

Occurrence.—Typical cholera is an acute disease of sudden onset, characterised by intense diarrhoea and tenesmus, vomiting, "rice-water" stools, muscular cramps and extreme collapse.

The vibrios multiply freely in the lumen of the small intestine and are present in large numbers in the intestinal contents and dejecta. There is great epithelial desquamation, and the stools contain white flakes consisting of epithelial cells and mucus. Numbers of vibrios are demonstrable in these flakes. The organism does not penetrate deeply in the bowel wall and practically never invades the blood stream. The gall bladder is frequently infected.

Convalescents may remain carriers, and the organism may persist in the gall bladder.

Cholera vaccine has been used in the prophylaxis of the disease. It is prepared from a 24 hours' culture on nutrient agar (*vide* p. 284) and the bacterial suspension is killed by heat at 55° C. (one hour) and then standardised. Two doses are given at intervals of seven days, 5000 and 10,000 million organisms respectively.

DIAGNOSIS

The bacteriological diagnosis depends on the isolation and identification of the vibrio.

The organism may be detected microscopically in the intestinal dejecta, but this is not sufficient for accurate laboratory diagnosis, and inexperienced workers may easily be misled by slightly curved bacilli simulating vibrios.

Procedure for Culture and Isolation

I.—(*a*) A tube of 10 c.c. peptone water is inoculated with a flake of mucus from the stool, or, in the case of a fluid faecal stool, with a large loopful of the specimen. In examining possible carriers, the stool, if solid or semi-solid, is thoroughly emulsified in sterile salt solution and several loopfuls are added to the medium. The peptone water used is a 1 per cent. peptone with 0·5 per cent. sodium chloride, standardised to pH 8·2. The tube is incubated for six to eight hours. Within this time vibrios, if present, grow freely and at the surface of the medium, and even outgrow other intestinal bacteria.

(*b*) A plate of Dieudonné's medium (*vide* p. 150) is also inoculated directly from the stool, and incubated for eighteen to twenty-four hours. This medium is highly selective for vibrios, inhibiting the growth of most other intestinal bacteria. A practically pure culture of an intestinal vibrio can often be obtained directly in this way from a stool containing large numbers of *B. coli.*

II. The peptone-water culture is examined after six to eight hours by means of a stained film made from a drop of the surface layer of the culture: a large loopful is placed on a slide and, without spreading, slowly dried at room temperature; the film is then fixed by heat, and washed in a stream of water to remove the dried peptone particles which stain deeply and obscure the organisms; the preparation is stained with dilute carbol fuchsin for one minute and examined microscopically. At the same time a hanging-drop preparation may be examined; at the edge of the drop, vibrios are easily detected by their characteristic morphology and "scintillating" or darting motility. In general, however, the fuchsin-stained film can be relied on alone for the detection of vibrios. If vibrios are present, a sub-inoculation is made on a Dieudonné plate. If no vibrios are detected, a sub-inoculation is made into a second peptone-water tube; this tube is incubated for six to eight hours, and a film from it is then examined as in the case of the primary culture: if vibrios are present, a Dieudonné plate is inoculated from the peptone culture. If no vibrios are detectable in the second peptone-water culture the result may be regarded as negative.

Where vibrios are relatively scanty, and are not cultured directly on a Dieudonné plate, the organism can be isolated after enrichment in either one or two peptone-water cultures.

Pure cultures on agar slopes are obtained from isolated colonies on the Dieudonné plates; the morphological, cultural and biochemical characters of the strains are then determined ; the final identification of the organism depends on its agglutination by a specific anti-cholera serum obtained by immunising an animal with a known *V. cholerae* (*vide* p. 473). In view of the fact that the H antigen of *V. cholerae* may be common to other vibrios, the test may be made more specific by using an O-agglutinating serum. If the antiserum available gives both H and O reactions, the bacterial suspension should be heated at 100° C. for 20 minutes to inactivate the H antigen.

If a direct growth from the stool is obtained on Dieudonné's medium it is, as a rule, practically pure, and the bacteriological diagnosis can be expedited by carrying out the agglutination reaction at once from the plate culture. At the same time it is essential to obtain cultures from single colonies and confirm the identity of the organism by detailed tests. It must be remembered that the El Tor vibrio is serologically similar to *V. cholerae* and that the haemolytic test (*vide supra*) is required for the differentiation of the two organisms.

ISOLATION OF VIBRIO CHOLERAE FROM WATER

100 c.c. of a sterile alkaline (pH 9·0) 10 per cent. solution of peptone containing 5 per cent. sodium chloride are added to 900 c.c. of the water specimen, which is then distributed in sterile stoppered flasks or bottles. These are incubated, and sub-inoculations are made (from the surface growths) on Dieudonné's medium after twenty-four and forty-eight hours, as in the method described above for the isolation of *V. cholerae.*

A larger quantity of water may be tested by filtering it through a Seitz disk (p. 88) and by using the disk as the inoculum for a peptone-water culture.

THE PARACHOLERA VIBRIOS

These are associated with choleraic conditions, usually of lesser severity than true cholera, and occurring as sporadic cases or in limited outbreaks.

Their morphological, cultural and biochemical characters generally correspond to those of *V. cholerae*, but the reactions with mannose and arabinose may differ from those of the cholera vibrio (*q.v.*). Most of the types described are haemolytic, like the El Tor vibrio (*vide supra*).

When tested in the form of plain saline suspensions they do not react specifically with an agglutinating anti-cholera serum, and represent a number of serological types, differing in their agglutination reactions with antisera.

These organisms may possess an H antigen in common with *V. cholerae* but their O constituents are distinct. The different serological races have distinctive O antigens.

The bacteriological diagnosis in paracholera is carried out in the same way as in true cholera.

OTHER VIBRIOS

Two well-defined species of vibrios have been described in diseases of animals—*V. metchnikovi* and *V. foetus*.

V. metchnikovi was first isolated from a septicaemic disease of fowls. It resembles closely *V. cholerae* in general biological characters, but differs serologically, and in its high virulence for guinea-pigs, pigeons and fowls: a minute amount of culture introduced intramuscularly or into a cutaneous wound produces in these animals a rapidly fatal septicaemia. *V. cholerae* does not show such degree of pathogenicity. Similar organisms have been isolated from choleraic cases and from water.

V. foetus has been reported in abortion of sheep and cattle, and isolated from the placenta and also from the foetus. The organism varies in length, the short forms being comma-shaped, the longer individuals exhibiting two to four coils. It is relatively slender and shows a flagellum at one or both ends. It stains Gram-negatively. This organism is micro-aerophile and has been cultivated on agar slopes in sealed tubes containing in the condensation water a few drops of sterile defibrinated horse blood. Growth is not abundant and is most marked in the condensation water. When first cultivated growth may only develop between the agar and the wall of the tube; after repeated subculturing a surface growth is obtained. Laboratory animals are not susceptible to experimental inoculation.

Vibrio jejuni has been described in an infectious diarrhoea ("winter dysentery") of cows in America. It is a Gram-negative vibrio with a flagellum at one or both poles and appears to be related to *Vibrio foetus*.

Various other vibrios and spirilla have also been described. These are mostly water forms. In certain parts of India, vibrios are regularly present in unprotected wells and rivers. These may present some similarity to the cholera vibrio but are serologically distinct and they occur in areas where cholera is not endemic. Many of them correspond to the vibrios described in paracholera though such water vibrios are apparently non-pathogenic. Certain water vibrios exhibit in culture marked phosphorescence—e.g. *V. phosphorescens*. Vibrios have been isolated from a variety of other sources—*e.g.* from sputum (*V. sputigenus*), from cheese (*V. tyrogenus*), from intestinal contents in "Cholera nostras" (*V. proteus*—Finkler and Prior's spirillum) and from infections in fish.

BACILLUS PESTIS (*Pasteurella pestis*)

The organism of Oriental Plague.

Morphology and Staining.—In its most characteristic form this organism is a short, oval bacillus with rounded ends—*i.e.* cocco-bacillary—about 1·5μ by 0·7μ and occurring singly and in pairs. In the tissues and in culture a typical capsule may be observed; in cultures grown at 37° C. an envelope has been demonstrated by means of India ink preparations.

It is Gram-negative, and when stained with a weak stain (*e.g.* methylene blue) shows characteristic bipolar staining which is an important feature in identification.

In culture the plague bacillus is less typical. Longer forms are frequent, and polar staining is less obvious. Pleomorphism is marked especially in old cultures, and involution or degeneration forms are particularly noticeable. These are markedly enlarged, stain faintly and include globular, pear-shaped, elongated or irregular forms. In fact the microscopic picture of an old culture often suggests that of a yeast or mould. Involution in culture can be hastened by the presence of 3 per cent. sodium chloride, and this has been utilised in identifying the organism.

In fluid culture the bacilli tend to be arranged in chains.

The organism is non-motile and non-sporing.

Culture.—Grows aerobically and anaerobically on ordinary culture medium. The optimum temperature of the plague bacillus, unlike other pathogens, is below 37° C., and primary cultures grow best at 27° C.. The minimum temperature is about 14° C..

B. pestis is somewhat sensitive to free oxygen and growth may not develop under aerobic conditions if the inoculum is small; this inhibition can be avoided by the addition of blood or sodium sulphite to the medium or by the exclusion of air.

Colonies on agar—at first very small, transparent, white, circular disks (1 mm. or less), later becoming larger (3–4 mm.) and opaque; they are not specially characteristic.

In older cultures some of the colonies may have outgrown the others and become more opaque. This appearance is not unlike that of a mixed growth.

Gelatin stab—no liquefaction occurs.

Broth—growth consists of a granular deposit at the foot and on the side of the tube, not unlike that of a streptococcus. If cultured in a flask of broth with drops of sterile oil on the surface, and provided the flask is not subjected to shaking or movement, a characteristic growth develops, consisting of "stalactites" hanging down into the fluid from the oil drops.

Biochemical Reactions.—

Glucose	Lactose	Dulcitol	Saccharose	Mannitol	Milk	Indole
⊥	-	-	-	⊥	-	-

(⊥=acid; no gas)

Growth occurs on a bile-salt medium—*e.g.* MacConkey's (*cf.* other members of the *Pasteurella* group).

It should be noted that the risk of laboratory infection from handling pathological material and cultures is considerable, and all manipulations should be carried out with the utmost care.

Viability.—The thermal death-point is about 55° C.. Dies within one to two days when subjected to drying. Laboratory cultures remain viable for long periods (*e.g.* months) if kept moist and at low temperatures.

Antigens.—It has been shown that *B. pestis* contains two types of antigen, one somatic and heat-stable, the other heat-labile at 100° C. and associated with the envelope which is formed in cultures growing at 37° C. (*vide supra*). The envelope antigen may be of importance in relation to the immunising properties of *B. pestis* vaccines: thus, a vaccine prepared from cultures grown at 37° C., in which the envelope is well developed, is stated to have greater immunising properties than from cultures grown at lower temperatures—*e.g.* 25°–30° C. as in the preparation of the Haffkine plague vaccine generally used in India (Schütze). Heating the culture for half-an-hour at 56° C. before addition of phenol does not affect the antigenic value of the resulting vaccine. Strains of *B. pestis* are serologically homogeneous.

Animal Inoculation.—The bacillus is pathogenic to monkeys, rats, guinea-pigs and other rodents, and plague is essentially an epizootic disease among wild rats and certain other rodent animals. A guinea-pig or white rat injected subcutaneously with a recently isolated culture dies in a few days, and at autopsy a marked local inflammatory condition is noted, with necrosis and oedema; the related lymph glands are also involved; the spleen is enlarged and congested and often shows small greyish-white areas in its substance; there is also septicaemia. The characteristic bacilli can be seen in large numbers in films from the local lesion, lymph glands, spleen pulp and heart blood. A similar condition is found in rats dying of epizootic plague (*vide infra*).

Rats and guinea-pigs can be successfully inoculated by applying infected material to a shaved area of skin or to a mucous membrane—*e.g.* of the nose.

Toxin.—Marked local and general toxic effects can be produced in animals by injection of dead cultures but culture-filtrates are practically non-toxic. The toxin is apparently of the intracellular type.

Occurrence in Human Lesions.—In *Bubonic Plague* the bacilli are present in large numbers in the affected lymph glands. When the bubo undergoes necrosis as the condition advances, they become less numerous, and may even disappear. Septicaemia may result, and then the bacilli can be detected in the blood during life by blood culture. *Post mortem* they are found in the spleen.

In *Pneumonic Plague* the bacilli are present in large numbers in the sputum and in the broncho-pneumonic areas in the lung.

In *Septicaemic Plague* the condition is a general infection without definitely localised lesions.

Infection.—Plague is epizootic in rats and certain other rodents. The infection is spread by rat fleas (e.g. *Xenopsylla cheopis*). The occurrence of bubonic plague in man is due to transmission of the infection from rats by the same agency. The mechanism of transmission is as follows :—The flea sucks blood (containing plague bacilli) from an infected animal ; the bacilli multiply in the stomach and proventriculus, which may become blocked with bacillary masses ; when the insect again bites and sucks blood, regurgitation takes place from the blocked proventriculus into the bite wound, and so inoculation results.

The time during which a flea remains infective—*i.e.*, the period of survival of the bacilli—depends on temperature and humidity. A temperature of about 50° F. (10° C.) and a high degree of humidity have been found to be the most suitable conditions. A temperature over 80° F. (27° C.) is unfavourable.

Pneumonic plague is communicated from person to person by infected secretion droplets from the respiratory passages. This form of the disease may be initiated from cases of bubonic plague in which the organisms localise in the lung and produce a pneumonic lesion.

Plague vaccine has been extensively used for prophylactic purposes, particularly the preparation known as Haffkine's vaccine. It is a four weeks' culture of *B. pestis* grown at 27° C. in a goat's-flesh-digest broth. The culture is killed by heat at 55° C. (15 minutes) and 0·5 per cent. phenol is added as a preservative. One dose of 4 c.c. is injected subcutaneously.

Living non-virulent cultures have also been employed as vaccines.

Therapy.—Sulphonamide compounds have been used with some success for the treatment of plague. Therapeutic results have also been obtained with antisera.

DIAGNOSIS

Bubonic Plague.—The bubo is punctured with a hypodermic syringe and exudate withdrawn. From this material films are made and stained with methylene blue and by Gram's method. The appearance of the characteristic bacilli showing bipolar staining is highly suggestive.

Cultures are also made on blood-agar, and single colonies are subcultured. The resulting growths are then available for further investigation.

Some of the exudate should also, if possible, be injected subcutaneously into a guinea-pig or white rat. If plague bacilli are present, the inoculated animal will die, showing at autopsy the appearances, etc., described above.

The cultures obtained may be tested as regards: biochemical reactions, involution on 3 per cent. salt-agar, chain formation in broth, and stalactite growth. The cultures can also be used for further animal inoculation experiments.

Pneumonic Plague.—The bacilli can be detected microscopically in the sputum, and for identification should be isolated in pure culture as in dealing with material from bubonic plague.

In carrying out animal inoculation with sputum, other virulent organisms may be present (*e.g.* pneumococcus); instead of injecting subcutaneously, successful inoculation with *B. pestis* can be effected by applying the material to the nasal mucosa, or to a shaved area of skin.

In septicaemic plague, the bacillus can be demonstrated and isolated by blood culture (*vide* p. 182).

DIAGNOSIS OF PLAGUE INFECTION IN WILD RATS

At autopsy the following appearances are noted :—enlargement of lymphatic glands, with periglandular inflammation and oedema, most frequently in the cervical glands owing to the fact that the neck is the common harbourage of fleas ; serous effusion in the pleural cavity ; enlargement of the spleen, which may show small white nodules in the pulp ; congestion and a mottled appearance of the liver ; congestion and haemorrhage under the skin and in the internal organs.

Films are prepared from the heart blood, the glands and spleen, and stained by Gram's method and with methylene blue. Cultures should also be made, and the isolation of the organism attempted by the usual methods. Guinea-pigs should be inoculated subcutaneously with an emulsion of the splenic tissue. In rats found dead of plague it may be difficult to demonstrate the bacilli microscopically or to isolate them in culture. Carcases in a state of decomposition may be heavily contaminated with other organisms which render the microscopic examination confusing and isolation difficult. Infection of a white rat or guinea-pig, by smearing the nasal mucous membrane or a shaved area of skin with material from the esions, should be carried out.

PASTEURELLA GROUP

The plague bacillus is only one species in a biological group (the *Pasteurella* group) which includes the organisms of "haemorrhagic septicaemia" in various animals, and *B. pseudo-tuberculosis rodentium.* These organisms and *B. pestis* all show a similarity in morphology, staining reactions, cultural and biochemical characters, but differ in certain features and in their parasitism and virulence to different animal species.

Strains of *Pasteurella* group organisms isolated from haemorrhagic septicaemia have been generally named according to the animal in which they occur and they will be described here according to this system, but they are probably all members of the same species differing, perhaps, in their parasitic adaptations to particular hosts.

Bacillus avisepticus (*P. aviseptica*) is the causative organism of "fowl cholera" and septicaemia in certain birds. In the blood the bacilli are present usually in considerable numbers and show characteristic bipolar staining. Under experimental conditions this organism is virulent to fowls, pigeons and rabbits, but in guinea-pigs the infection may remain localised to the site of inoculation.

Bacillus suisepticus (*P. suiseptica*).—The organism of swine plague which takes the form of a rapidly fatal septicaemia or a pneumonia with enteritis. Various animals are susceptible to experimental inoculation—*e.g.* swine, rabbits, cats, cattle, sheep ; guinea-pigs and pigeons are stated to be less susceptible. This type of organism has been noted by various observers as a commensal in the respiratory passages of swine, and the question whether it is the primary causal agent of swine plague is not entirely settled.

Bacillus bovisepticus (*P. boviseptica*). The organism of haemorrhagic septicaemia of cattle and of septic pleuro-pneumonia of calves. On experimental inoculation this organism is highly pathogenic to cattle, rabbits and mice.

Other recognised organisms of the group are :—

B. lepisepticus (or *P. cuniculicida*) of rabbit septicaemia and snuffles (*vide* p. 245), *B. equisepticus* (of haemorrhagic septicaemia in horses). Similar infections also occur in sheep, goats, etc.. *B. pseudo-tuberculosis rodentium* occurs in pseudo-tuberculosis and septicaemia of rats and certain other rodents.

The organisms of haemorrhagic septicaemia can be differentiated from *B. pestis* by the inhibition or absence of growth on a taurocholate medium (*e.g.* MacConkey's) on which the plague bacillus is able to grow. *B. pseudo-tuberculosis rodentium*, which might be confused with *B. pestis* if isolated from wild rats, can be distinguished by its motility when growing at 22° C. and its feeble pathogenicity to white rats.

Different serological types of *B. pseudo-tuberculosis rodentium* have been recognised by agglutination reactions. The organism possesses three antigenic constituents : (1) flagellar, (2) somatic and type-specific, and (3) somatic and common to the different types. This last is the same as the somatic antigen of *B. pestis*.

The following Table shows how *B. pestis*, *B. pseudo-tuberculosis rodentium* and *B. avisepticus* (representing the haemorrhagic septicaemia subgroup) may be differentiated :

	Motility at 22° C.	*Saccharose*	*Milk*	*Indole*	*Growth on Bile-salt Media*	*Pathogenicity to White Rat*
B. pestis .	−	−	No change	−	+	+
B. pseudo-tub. rodentium .	+	$\underset{-}{\perp}$	Alk.	−	+	−
B. avisepticus	−	⊥	No change	+	−	+

In freshly isolated culture *B. pestis* can be differentiated from *B. pseudo-tuberculosis rodentium* and other *Pasteurella* group organisms by adding very small inocula (from dilutions of the culture) to rabbit-blood-agar and incubating at 37° C. : *B. pseudo-tuberculosis rodentium* grows well in twenty-four hours while *B. pestis* develops slowly at this temperature, small colonies appearing only after forty-eight hours. The optimum temperature for *B. pestis* when freshly isolated is 27° C. (*vide supra*).

The name *Pasteurella multocida* has been proposed for all the typical organisms of haemorrhagic septicaemia, the term *Pasteurella haemolytica* being applied to atypical strains of bovine or ovine origin which are avirulent and differ from the typical strains in their haemolytic properties and inability to produce indole. *P. multocida* has been subdivided by fermentative and agglutination reactions into two main subgroups with possibly a third intermediate subgroup. Group I comprises all strains of avian origin ; morphology is uniform ; the colony presents a fluorescent appearance ; growth in broth exhibits a uniform turbidity ; arabinose and dulcitol are usually fermented but not xylose. Group II contains strains from a variety of animals ; morphology is less regular ; colonies are mostly non-fluorescent ; growth in broth is of a mucoid type ; xylose is fermented but not arabinose and dulcitol (see Rosenbusch, C. T., and Merchant, I. A., *J. Bact.*, 1939, **37**, 69).

Rare cases have been recorded of human infection by organisms corresponding to the Pasteurellae of haemorrhagic septicaemia and rodent pseudo-tubercle.

BACTERIUM TULARENSE (*Pasteurella tularensis*)

In the Western States of America a plague-like disease occurs in wild rodents (*e.g.* rabbits, hares, ground-squirrels, etc.) due to an organism designated *Bacterium tularense.* The pathological lesions are not unlike those found in plague-infected animals, and this infection has to be considered, therefore, in the diagnosis of plague in animals. The disease has also been observed in Japan, Russia, Norway and certain other parts of Europe. Various rodent and other wild animals may be infected. The organism is a small rod-shaped structure not usually exceeding 0·7μ in length, and sometimes capsulated. It is present in large numbers in the spleens of infected animals, but cannot be cultivated like the plague bacillus on ordinary media. Cultures have been obtained on a medium consisting of pure egg yolk, on blood-agar and serum-agar containing a piece of sterile rabbit spleen and on horse-serum-agar containing 0·1 per cent. cystine and 1 per cent. glucose.

This organism shows serological relationships to the *B. melitensis-abortus* group.

This infection is also transmissible to man — *e.g.* from handling infected animals (*e.g.* rabbits and hares), and from laboratory cultures (*which retain an extremely high degree of infectivity*). A prolonged febrile illness results, sometimes with glandular lesions and ulcers of the skin. The serum of infected persons agglutinates the organism. For diagnostic purposes guinea-pigs or mice may be inoculated with exudate from the glands or ulcers.

Infection is also spread by ticks and other biting arthropods, and *Bacterium tularense* has been cultivated from ticks. The disease is sometimes apparently water-borne; thus, water-rats may be infected and contaminate water by their excreta.

CHAPTER XVII

BRUCELLA GROUP; HAEMOPHILIC BACTERIA (BACILLUS INFLUENZAE, ETC.); BACTERIUM PNEUMOSINTES

BRUCELLA GROUP

THE generic name *Brucella* is now applied to a group of pathogenic bacteria which include the organism of Malta fever (*Bacillus*, or *Brucella*, *melitensis*) and that of bovine contagious abortion (*Bacillus*, or *Brucella*, *abortus*). These organisms have generally been classified as separate species but in many of their features present a close similarity, and additional *Brucella* types can be recognised which seem to be intermediate biologically between them, *e.g.* the "porcine type" of *B. abortus* which is now frequently designated *Brucella suis*.

Certain animals (*e.g.* goats, sheep, cattle and pigs) are the natural hosts of these organisms, and abortion is an outstanding result of infection though an infected animal may often show no recognisable illness. The main *Brucella* types differ in their habituation to certain animal species: *B. melitensis* occurs usually in goats and sheep; the two types of *B. abortus* in cattle and pigs respectively. Each of these may produce undulant fever in man though the severity varies with the type: *B. melitensis* is more infective and causes a more severe illness than the bovine type of *B. abortus*, while the porcine type approaches *B. melitensis* in its virulence to the human subject.

BACILLUS MELITENSIS (*Brucella melitensis*)

The causative organism of undulant fever of the Mediterranean littoral and islands (Malta Fever), France, India, China, South Africa and certain areas of North and South America.

Morphology.—A cocco-bacillus, usually appearing as round or oval forms about 0·4μ in diameter. Definite bacillary forms (1–2μ in length), however, may be observed. The organisms occur singly, in pairs, or even short chains. It is non-motile and non-sporing.

Staining.—Gram-negative.

Culture.—Grows under ordinary aerobic conditions. Optimum temperature—37° C.. Grows even at 20° C.. It can be cultivated on ordinary nutrient media, but a better growth is obtained on liver-infusion agar (pH 6·6–6·8)—*vide* p. 150.

Colonies on agar in primary growth may not appear for two or three days; they are small transparent disks about 1 mm. in diameter, but increase in size to about 3 mm.

Gelatin stab—a delicate line of growth along the needle track with little or no surface growth. No liquefaction occurs.

On potato medium a greyish or brownish growth is noted.

B. melitensis exhibits no fermentative properties demonstrable by the ordinary methods.

Viability.—The thermal death-point is about 60° C.. Resists drying for two to three months. Laboratory cultures remain viable for several months.

Animal Inoculation.—Laboratory animals are relatively resistant to experimental inoculation, but if a large dose of culture is injected intramuscularly in the guinea-pig, infection may be produced; this is not progressive and does not lead to a fatal result as a rule. If the animal is killed after about two months and an autopsy carried out, necrotic areas are found in the liver and spleen in which the living organisms are present.

Occurrence.—The organisms are present in the blood, especially at an early stage. In some cases (about 10 per cent.) they may be demonstrated in the urine. *Post mortem* they are found in considerable numbers in the spleen and also in various organs. In Malta and the Mediterranean littoral, infection results usually from the ingestion of the milk of infected goats and the organisms can be demonstrated in the milk of a considerable proportion of goats in the endemic areas. Sheep may also be infected naturally with *B. melitensis*. In France both sheep and goats constitute reservoirs of the infection though cows also may carry the organism. In that country, cases of undulant fever occur mostly in the rural population and among persons who come into contact with infected animals or their carcases. Thus, the human infection may result either from the ingestion of milk or by contact with animals. It may be noted that recently *B. melitensis* infection of cattle has been reported in England.

A " rough " variant of *B. melitensis* can be recognised differing from *B. melitensis* in agglutination reactions with antisera, though otherwise similar. This type was originally designated " *B. paramelitensis.*"

DIAGNOSIS

Blood Culture should be carried out in all cases, and it is essential that at least 10 c.c. of blood should be withdrawn for this purpose, as the organisms may be relatively scanty. Cultures may also be obtained by spleen puncture.

In some cases the organism may be isolated from the urine.

The agglutination test with patient's serum and a known strain of *B. melitensis* is carried out as a routine procedure. The reaction may be elicited after five days from the onset of the illness. It has to be noted that apparently normal serum may agglutinate *B. melitensis* in low dilutions, *e.g.* 1 in 25. In cases of undulant fever, however, the serum often agglutinates *B. melitensis* in high dilutions—*e.g.* 1 in 1000. In a suspected case, if the reaction occurs only with low dilutions, the result cannot be regarded as conclusive. When the test is repeated, a " rising titre " may be observed and a more conclusive result obtained.

Post mortem, the organism can be cultured from the spleen.

In goats, the infection can be recognised by using the agglutination test with the animal's serum and by cultivating the organism from the milk.

BACILLUS ABORTUS (bovine type—*Brucella abortus*; porcine type—*Brucella suis*)

The organism of bovine contagious abortion; occurs also in pigs and has been observed occasionally in other animals, *e.g.* sheep, horses, fowls. In morphology and general biological characters this organism closely resembles *B. melitensis* and may cause undulant fever in the human subject.

Two types were originally recognised, *bovine* and *porcine*, these being the types of *Brucella* commonly found in cattle and pigs respectively. The *porcine* type, however, resembles *B. melitensis* in certain important features and is now designated frequently under a separate specific name, *Brucella suis*.

When cultivation is attempted directly from the animal body, the *bovine* type does not grow under ordinary aerobic conditions and requires an atmosphere containing 5–10 per cent. of carbon dioxide. This can be obtained by placing the inoculated tubes or plates in an air-tight jar, exhausting it to the required degree and replacing 5–10 per cent. of the air with carbon dioxide (*vide* p. 178). After continued cultivation, however, the organism may be grown in the ordinary atmosphere. The *porcine* type, like *B. melitensis*, can be grown under the usual aerobic conditions and is not dependent on such a high carbon dioxide content in the atmosphere as the *bovine* form. This is an important distinguishing feature. Bang originally cultivated the *bovine* type by preparing shake cultures in tubes of serum-agar, the colonies developing best in a zone just below the surface of the medium. This is due to the fact that in this zone the partial pressure of carbon dioxide is at an optimum for the growth of the organism. A convenient method of producing a suitable atmosphere for the growth of this organism in a tube is simply to seal the mouth of the tube with paraffin-wax after flaming the stopper. This procedure usually results in a sufficiency of carbon dioxide in the contained air.

The most suitable medium is a liver-infusion agar (*vide* p. 150); the addition of gentian-violet in a concentration of 1 : 250,000 facilitates isolation of the organism from material likely to contain other bacteria.

On potato medium *B. abortus* produces a brown growth like that of *B. melitensis*; it exhibits no obvious fermentative reactions.

Both the *bovine* and the American *porcine* type form sulphuretted hydrogen (the latter more markedly), which is not a property of *B. melitensis* (see table, p. 492). This can be tested for by placing a piece of moistened lead acetate paper at the mouth of the culture tube and replacing it, if necessary, daily for five days. The *porcine* type found in Denmark does not produce sulphuretted hydrogen.

B. melitensis and the *bovine* and *porcine* types of *B. abortus* have been differentiated by means of media containing 1 : 25,000 basic fuchsin and 1 : 30,000 thionin respectively. *B. melitensis* is not inhibited to any extent by these dyes, the *porcine* type is inhibited by fuchsin but not by thionin, whereas the *bovine* type is inhibited by thionin, not by fuchsin (see table, p. 492).

	CO_2 requirement	*H_2S production*	*Growth in presence of*	
			Basic fuchsin 1:25,000	*Thionin* 1:30,000
B. melitensis . .	–	–	+	+
B. abortus bovine type . .	+	+	+	–
B. abortus porcine type (American strains) .	–	++	–	+

B. abortus can be isolated from the stomach contents, heart blood and tissues of the aborted foetus and from the uterine exudate, and in such materials it may also be demonstrated microscopically by appropriate methods. It may be present in the udder and excreted in the milk. Its specific relationship to the disease has been established by the experimental production of abortion in pregnant animals following intravenous injection of cultures or inoculation into the vagina. Similar experimental effects have also resulted from administration of cultures by the mouth.

Experimental inoculation of cultures into guinea-pigs produces a non-lethal infection with tubercle-like lesions (*e.g.* in lymph glands, spleen, liver, etc.), and as *B. abortus* may occur in cow's milk, this has to be remembered in relation to the animal inoculation test for tubercle bacilli (*vide* p. 310).

Strains of *porcine* type are more virulent for guinea-pigs than those of *bovine* origin.

The *bovine* type of *B. abortus* is less virulent to monkeys than *B. melitensis* which produces under experimental conditions in these animals a condition analogous to undulant fever. The *porcine* type resembles *B. melitensis* in its virulence for monkeys.

An "R" variant of *B. abortus* may occur in culture. This variant was originally designated "*B. para-abortus.*"

Diagnosis in animals.—The agglutination test with the serum of supposed infected animals and known cultures of

B. abortus has been used in diagnosis. Results in which agglutination occurs in dilutions of 1 in 20 or over are accepted as positive.

A convenient method of diagnosis is by means of the *whey-agglutination* test. Milk from each of the four quarters of the udder is mixed and clotted with rennin. The separated whey is then tested for agglutination of *B. abortus* in the same way as serum. A titre of 1 in 80 or over is usually diagnostic of udder infection.

In animals that have aborted, the organism can be demonstrated microscopically in the uterine discharge and also in the stomach contents of the foetus, and can be cultivated by the methods referred to above. Inoculation of a guinea-pig may be resorted to for demonstrating and isolating the organism ; the inoculated animal is killed after four weeks and cultures are made from the spleen. The inoculation test is also utilised for demonstrating the organism in milk.

Immunisation.—Certain avirulent strains of *B. abortus* used in the living state as vaccines have been shown to produce an effective immunity against contagious abortion and are applied practically in controlling the disease in herds.

RELATIONSHIP OF B. ABORTUS TO B. MELITENSIS AND ITS OCCURRENCE IN UNDULANT FEVER

B. melitensis and *B. abortus* show a very close biological relationship. Direct agglutination tests with antisera fail to distinguish between them. Agglutinin-absorption tests, however, elicit a difference. This difference in the antigenic constitution of the two organisms is quantitative rather than qualitative. Thus, both possess two similar antigenic constituents though in different proportions, one constituent being dominant in *B. melitensis* while the other predominates in the bovine type of *B. abortus.* In antigenic characters the American *porcine* type occupies a position intermediate between *B. melitensis* and the *bovine* type of *B. abortus.*

It is now well established that *B. abortus* may, under certain conditions, produce undulant fever (" abortus fever ") in man, infection being derived from cow's milk, or through contact with cattle or pigs ; thus strains of *porcine* origin as well as *bovine* strains may be responsible for human infections. *B. abortus* may occur in a considerable percentage of samples of unsterilised market milk, but the incidence of " abortus fever " in man is exceedingly low. Latent infections, however, are not uncommon. It would appear, therefore, that the infectivity of the *bovine* strains of *B. abortus* must be comparatively slight for the human subject. The *porcine* type, with the exception of the Danish strains, possesses a higher virulence. *B. abortus* infection in man may be either a typical " undulant fever " or an irregular febrile illness which is mild in type. The serum of these cases agglutinates *B. abortus*, often in high dilutions, and the organism may be cultivated from the blood.

A type of *B. abortus* has been reported in undulant fever in Rhodesia; this organism, however, resembles *B. melitensis* in its ability to grow in the ordinary atmosphere and in its virulence for man. The infection is derived from cattle.

In the South-east of France *Brucella* strains have been isolated with the biological characters of *B. abortus* but the antigenic structure of *B. melitensis*. In pathogenicity they resemble *B. abortus*.

B. bronchisepticus, an organism originally described in canine distemper, is biologically related to *B. melitensis* and *B. abortus*. It differs from these organisms in being motile and possessing peritrichous flagella. It represents a secondary infection in distemper. This organism is also found in " snuffles " of rabbits.

BACILLUS INFLUENZAE AND THE HAEMOPHILIC BACTERIA

Bacillus influenzae, originally described as the causal organism of epidemic influenza, has been designated " haemophilic " in virtue of its inability to grow on culture medium without the addition of whole blood or certain growth-promoting substances present in blood (*vide infra*). These growth factors, however, are not restricted to blood, but are present also in certain vegetable tissues. The Koch-Weeks bacillus (*vide* p. 352) shows the same growth requirements as *B. influenzae*, and may appropriately be grouped with it. *B. pertussis* (of whooping-cough) has also been placed in the " haemophilic " group, but its growth requirements are entirely different from those of the influenza bacillus. Similarly the bacillus of Morax (*vide* p. 353) and Ducrey's bacillus (*vide infra*) have been included by some systematists in the " haemophilic group " along with *B. influenzae*, but such grouping is not strictly justifiable. If the term " haemophilic " is used in a broad sense to designate organisms which require blood for their growth it would embrace a number of heterogeneous species, and it is preferable to restrict the term to those organisms which are dependent on the growth factors required by *B. influenzae*.

BACILLUS INFLUENZAE (PFEIFFER)
(*Haemophilus influenzae*)

Morphology.—A very small slender bacillus, usually about 1·5μ by 0·3μ, with rounded ends, occurring singly or in pairs; non-motile; non-sporing. Shorter oval cocco-bacillary forms are also noted, and in culture there is marked pleomorphism. Some strains, particularly those present in meningitis, show elongated curved thread-like forms.

Staining.—Gram-negative; carbol fuchsin in a 1 in 20 dilution should be used as the counter-stain and applied for five minutes.

Culture.—Aerobe. Optimum temperature — about 37° C.. Does not grow on ordinary media but can be cultivated in the presence of blood—therefore classified as " haemophilic "—*e.g.* on blood-agar or preferably heated-blood-agar (*vide* p. 134).

It has been shown that two growth-promoting constituents present in blood are necessary for the cultivation of *B. influenzae.* One of these, termed the X factor, is thermostable and resists autoclaving at 120° C.. This factor is haematin and is supposed to act in virtue of its being required for the synthesis of catalase which is necessary for the aerobic growth of the organism (*vide* p. 172). It has been found that the X factor can be dispensed with under anaerobic conditions. It is claimed also that cysteine can replace haematin under aerobic conditions since in the presence of this substance hydrogen peroxide would be reduced and catalase would not be required. The other factor, designated V, is more easily destroyed by heat. It has been identified as coenzyme I (cozymase). It should be noted that coenzyme I is widely distributed in plants and animals and may be considered part of the essential equipment of all living cells.

On blood-agar—very small transparent droplet-like colonies which tend to remain discrete. In culture transformation to the "rough" type of colony may be observed.

It is noteworthy that *B. influenzae* grows better in symbiosis with staphylococci, etc.. This is due to the fact that these organisms synthesise the V factor. Thus, in a mixed culture the growth of the influenza bacillus is more marked in the neighbourhood of staphylococcal colonies: this appearance has been designated "satellitism."

Glucose and various other carbohydrates are fermented. Indole is produced by some strains.

Haemolytic types.—Certain strains classified as *B. influenzae* have been found to differ from the typical form in their haemolytic properties and these are sometimes coarser in microscopic appearance. They also tend to develop elongated threads. While the typical influenza bacillus requires for its growth both the X and V factors, most of these haemolytic strains require only the V factor.

Some non-haemolytic strains also are independent of the

X factor and require only the V substance. These have been designated "*Para-influenza bacillus.*" Strains of this type have been described in ulcerative endocarditis.

B. haemoglobinophilus canis, an organism originally isolated from a purulent condition of the preputial sac in a dog, resembles *B. influenzae* in general characters, but requires only the X factor for its growth.

A somewhat similar organism has also been described recently in endocarditis of the human subject. This type of *Haemophilus* requires carbon dioxide for its growth.

B. influenzae suis.—This organism has been found in an influenza-like disease of pigs associated with a filterable virus (*vide* p. 623). It differs from *B. influenzae* in the lack of fermentative action.

Experimental Inoculation.—It has not been found possible to produce in laboratory animals a condition corresponding to influenza by experimental inoculation with this organism. According to certain observers a soluble toxin is formed in culture which on inoculation in laboratory animals produces pulmonary lesions like those in epidemic influenza.

Occurrence.—Found in the sputum, nasal and throat secretions in a considerable proportion of cases of epidemic influenza with inflammatory conditions of the respiratory system. In the inflammatory exudate the bacilli are often seen inside leucocytes. In the sputum they may occur in exceedingly large numbers, but usually along with the pneumococcus, streptococci and other organisms associated with inflammation of the respiratory passages. They may be found in the pulmonary lesions in influenzal pneumonia, in empyema and other complications, and have also been noted in the blood in some cases.

It must be remembered that *B. influenzae* may occur in catarrhal conditions of the respiratory system *apart altogether from epidemic influenza*, and it may also occur in the mouth and throat in healthy persons.

To facilitate the isolation of *B. influenzae* from sputum, etc., "*penicillin*," an antibiotic product of *Penicillium notatum* has been used (Fleming). In culture medium it permits the growth of the influenza bacillus while inhibiting that of various Gram-positive cocci.

Penicillin was originally prepared by the following method: *Penicillium notatum* is grown for ten days in broth at 22° C.; the maximum yield is after five to ten days' incubation; on and after the fifth day samples are withdrawn daily and the filtrates diluted in broth in series from 1 in 100 to 1 in 500; these are inoculated with one drop of a broth culture of a staphylococcus; when the filtrate in a dilution of 1 in 250 or higher, placed on the surface of culture medium, inhibits the growth of the staphylococcus, the whole culture is filtered through a Seitz disk; the filtrate is neutralised with 2·5 per cent. sulphuric acid until phenol-red indicator just turns yellow (pH 6·8); the filtrate is stored in the refrigerator but soon commences to deteriorate, and may be inactive within a month. For the selective cultivation of *B. influenzae* the penicillin-filtrate is added to the surface of the medium to be used.

More recently improved methods have been devised for obtaining the maximum yield of penicillin from cultures and extracting this substance in the purest and most concentrated form possible so that it can be applied as a therapeutic agent in certain bacterial diseases (see **Appendix**).

Pfeiffer's influenza bacillus cannot be regarded as the specific primary cause of epidemic influenza, though it may play an important part in the pathogenesis of the pulmonary complications. Its rôle is a secondary one.

Haemolytic strains (*vide supra*) have been found in cases of meningitis occurring in young subjects, mostly between the ages of six months and one year.

Antigenic structure.—Recently isolated "S" forms of *B. influenzae* have been classified into two main serological types possessing specific carbohydrates as capsular constituents. In culture the organism readily undergoes transformation to the "R" type which lacks such specific substance, and under these conditions strains assume considerable serological diversity.

BACILLUS PERTUSSIS (*Haemophilus pertussis*)

Generally accepted as the causative organism of whooping-cough (pertussis).

Morphology and Staining.—A very small oval coccobacillus, slightly larger than *B. influenzae*, often showing polar staining; definite bacillary forms are noted, but it is generally more oval in form than *B. influenzae*, and is more uniform in size and shape; non-motile; non-sporing; Gram-negative.

Culture.—Strict aerobe. Compared with *B. influenzae*, it is not so strictly "haemophilic." It is usually first cultivated on media containing a large proportion of fresh blood: in subculture, however, growth may be obtained on media containing serum but without blood pigment, and it is independent of the X and V factors required by the influenza bacillus. For primary culture the special medium of Bordet and Gengou or a modification of this (p. 149) should be employed. The colonies develop slowly, and are smaller but thicker and more opaque than those of *B. influenzae*, and have a "pearly" or "mercury drop" appearance; growths may become sticky or slimy. Stroke subcultures have been likened to "streaks of aluminium paint."

The organism has no fermentative properties.

B. pertussis differs also from the influenza bacillus in its continued viability at low temperatures (0°–10° C.) and its inability to grow, when first isolated, on agar containing *heated* blood.

Experimental inoculation.—Intranasal inoculation of *B. pertussis* cultures in mice anaesthetised with ether produces an interstitial pneumonia which may be fatal. Young rats can be infected readily in the same way. In these animals paroxysmal coughing may occur and continue for a considerable period if the infection is not fatal. A condition similar to clinical whooping-cough has been produced in monkeys by introduction of cultures into the respiratory tract.

B. pertussis forms a potent *endotoxin* which can be extracted from cultures. This toxin is lethal to guinea-pigs on intravenous injection. In rabbits a dermo-necrotic action can be demonstrated.

Serological properties.—Recently isolated strains appear to be identical in antigenic characters and react similarly with agglutinating and complement-fixing antisera. After artificial cultivation, however, antigenic variation occurs, associated with changes in colony characters analogous to the S⟶R transformation of other bacterial species.

Occurrence.—Present in sputum, especially in the early stages. It would appear from the evidence available that *B. pertussis* is the specific causal agent of whooping-cough.

It may be noted here that lymphocytosis is characteristic of this disease.

Bacillus pertussis vaccines.—There is now considerable evidence that vaccination confers increased resistance to the disease and this procedure is advocated for prophylaxis in young children. A form of vaccine which has been successfully employed is a suspension of recently isolated cultures of the organism (in the S phase) killed by 0·5 per cent. phenol. The vaccine is standardised to contain 10,000 million organisms per c.c. and is injected in graduated doses amounting in all to 7–8 c.c. Even larger doses have been recommended, *e.g.* 6 c.c. of a vaccine containing 20,000 million organisms per c.c.

DIAGNOSIS

For detecting and isolating the organism the "cough-plate" method may be used: a plate of the Bordet-Gengou medium is held four inches in front of the mouth of the patient while coughing; the plate is thus inoculated directly with the droplets of sputum. After three days' incubation the "pearly" or "mercury drop" colonies of *B. pertussis* can frequently be recognised without difficulty, and identified by microscopic, cultural and serological methods.

In using this "cough-plate" method it is essential to avoid over-inoculating the plate; otherwise the medium becomes overcrowded with colonies and *B. pertussis* is then difficult to detect and isolate; moreover certain other organisms present —*e.g.* staphylococci—may inhibit growth of *B. pertussis*. Generally a plate with not more than 100 colonies is suitable. It is recommended that two plates should be exposed during the spasm. Penicillin as used for the isolation of *B. influenzae* (*vide* p. 498) is of value in controlling the growth of staphylococci on the plates and may even yield a pure growth of *B. pertussis*. When colonies with the characters of *B. pertussis* develop on the medium these are spread with an inoculating wire and the plate is re-incubated. From this larger quantity of growth a nigrosin preparation (p. 197) is examined and if the organisms show the characteristic morphology some of the growth is emulsified in a drop of saline on a slide, and a drop of anti-pertussis rabbit serum appropriately diluted is added. Agglutination of the organism confirms the diagnosis. A patient yielding two negative results by the cough-plate method in a week may be regarded as non-infective.

A convenient alternative method of obtaining a specimen for diagnosis is by using the post-nasal swab (see **Appendix**).

Serum diagnosis.—Both complement-fixation and agglutination tests have been applied. Such reactions are likely to be most pronounced at a later stage of the disease, when the organisms are less easily demonstrated in the sputum, and may be useful in corroborating the diagnosis of atypical cases.

BACTERIUM PNEUMOSINTES (*Dialister pneumosintes*)

This organism was first isolated from the nasopharynx of cases of epidemic influenza.

Morphology and Staining.—A minute cocco-bacillary organism measuring 0·15–0·3 μ in length. After prolonged artificial culture, its length may attain 0·5–1 μ. The organisms are usually found singly, but may be in pairs, and even in short chains of three or four individuals. *Bacterium pneumosintes* is stained best with well-ripened polychrome methylene blue, when the organism appears deep purple in colour. It is Gram-negative.

In young cultures a certain proportion of the organisms are small enough to pass Berkefeld V and N filters.

Culture.—Obligatory anaerobe. Optimum temperature—37° C.. Grows well in Smith-Noguchi medium (p. 151), forming a haze in the medium around the fragment of kidney in three to four days. On anaerobic blood-agar plates minute transparent colonies are formed after about six days' incubation.

Experimental Inoculation.—It has been stated that young cultures inoculated intratracheally in rabbits produce slight fever, and leucopenia due to a diminution of mononuclear cells. It is not fatal for these animals.

Occurrence.—Was originally found in the nasal washings of epidemic influenza in the first thirty-six hours of the disease. Similar organisms have been observed in the throats of healthy persons and in other pathological conditions of the respiratory passages.

It is doubtful whether this type of organism has any pathological relationships.

BACILLUS OF DUCREY (*Haemophilus ducreyi*)

Associated with " Chancroid " or " Soft Sore."

Morphology.—A rod-shaped organism 1·5μ by 0·4μ ; occurs in pairs and chains ; present in the exudate from the sore, in the tissue lesion and in the secondary buboes ; it is non-motile and non-sporing.

Staining.—Gram-negative.

Culture.—This organism has proved difficult to cultivate artificially, and appears to be a strict parasite. It has been found, however, that primary cultures can be obtained from the sore by inoculating directly tubes containing coagulated rabbit blood. These are prepared by distributing fresh rabbit blood, withdrawn from an ear vein or by cardiac puncture, in amounts of 1–2 c.c. in small test-tubes. The tubes are sloped and when the blood has clotted are heated at 55° C. for five minutes. The inoculum is introduced into the serum which has separated from the clot. After growth in these, the organism can be isolated on blood-agar plates. This method has been used for diagnostic purposes.

To obviate the difficulty arising from the presence of other organisms in the exudate of the sore, it has been recommended that the exudate should be injected intracutaneously in the forearm of the patient ; after three days, culture-medium is inoculated from the resulting lesion.

Pure cultures can be obtained by puncturing a bubo with a syringe, drawing up some of the pus, and with this inoculating tubes of coagulated blood.

An allergic skin reaction, produced by the intracutaneous injection of killed culture, has been utilised for diagnosis and has been regarded as specific.

Haverhillia multiformis (*Streptobacillus moniliformis*)

This organism was reported in France in cases diagnosed as erythema multiforme, and named *Streptobacillus moniliformis* ;

it was also described in America in a group of cases of multiple arthritis associated with an erythematous eruption (Haverhill Fever—Erythema arthriticum epidemicum). It has been found associated with a disease of mice characterised by multiple arthritis often involving the joints of the feet and leading to swellings of the feet and legs. The organism may occur in the nasopharynx of rats, and some cases of "rat-bite fever" are due to it.

The organism is a Gram-negative, pleomorphic bacterium, occurring as short rod-shaped forms ($1–3\mu \times 0{\cdot}3–0{\cdot}4\mu$) or as elongated filaments which may show characteristic fusiform enlargements. Growth can be obtained in the presence of blood, serum or ascitic fluid, and a high proportion of blood or serum is required in the medium. Löffler's serum medium serves well for cultivation. The colonies are small (1 mm.). Viability in culture is feeble and cultures die in two to four days.

Mice are susceptible to experimental inoculation and develop either a rapidly fatal general infection without focal lesions or a more slowly progressive disease with swelling of the feet and multiple inflammatory lesions of joints.

In the human infection the organism has been isolated by blood culture, and from joint fluid in cases with arthritis.

CHAPTER XVIII

TETANUS BACILLUS; OTHER ANAEROBIC BACILLI OF INFECTED WOUNDS; BACILLUS BOTULINUS; BACILLUS CHAUVOEI

BACILLUS TETANI (*Clostridium tetani*)

THE causative organism of tetanus.

Morphology.—Straight, slender, rod-shaped organism 2–5μ by 0·4μ, with rounded ends; shorter forms and longer filaments are also noted; motile, due to numerous long wavy flagella, peritrichous in arrangement, but movement is not markedly active. Characteristic spores are developed—spherical, two to three times the diameter of the bacillus, and situated terminally, they produce the "drum-stick" appearance which is a striking morphological feature of the organism.

Staining.—Gram-positive, but different individuals may show variation in the reaction to Gram's stain; by the ordinary staining methods, only the periphery of the spore is stained.

Culture.—An obligatory anaerobe, but growths can be obtained in the presence of minimal traces of oxygen; temperature range—14°–43° C., optimum—37° C.; grows on ordinary nutrient media; glucose is often added to culture media as a reducing agent; the meat medium described on p. 153 and media prepared from a peptic digest of blood (p. 508) are suitable for the growth of the tetanus bacillus. For methods of anaerobic cultivation see p. 172.

On agar or blood-agar, surface colonies of the normal motile type of tetanus bacillus are characterised by their long branching projections. After forty-eight to seventy-two hours' incubation the central part of the colony, which rarely grows more than 1 mm. in diameter, becomes slightly raised and has a "ground-glass" appearance, while the edge shows a feathery appearance, and the whole growth may spread over the surface of the medium by means of the branching processes from the colonies.

Non-motile variants may produce quite isolated colonies without these characteristic feathery processes.

Agar stab culture—no growth occurs on the surface; a white line of growth appears along the needle track but stops short of the surface, and lateral spikes, which are longest in the deeper part of the tube, develop from the central growth.

Milk—usually no coagulation.

Gelatin—is slowly liquefied.

Coagulated serum—is rendered more transparent and softened but is not liquefied.

Meat medium—gas production but only slight digestion and blackening of meat.

No carbohydrates are fermented.

Cultures have an unpleasant odour which is not markedly putrefactive.

Viability of Spores.—The spores possess a high resistance to adverse agents. They may withstand boiling water for forty to sixty minutes, and even longer. They may resist dry heat at 150° C. for one hour, 5 per cent. phenol and 1:1000 perchloride of mercury for considerable periods (two weeks or more). Iodine in watery solution and hydrogen peroxide (10 volumes) kill them within a few hours.

The *toxin* is an exotoxin and can be prepared artificially by growing the organism in broth and filtering through an earthenware filter after five to fourteen days' growth, the optimum time varying with the strain. There are two constituents—*tetanospasmin*, which acts on the nervous system, and also *tetanolysin*, which causes lysis of red blood corpuscles.

Tetanus toxin is an extremely powerful poison. When injected into guinea-pigs or mice the animals die in twelve to eighteen hours with the typical signs of tetanus. In animals tetanic spasms may start in the muscles related to the site of injection ("local tetanus"). It has long been supposed on the basis of experimental results that the toxin reaches the central nervous system by passing along the motor nerves, being absorbed probably by the motor end-plates and spreading up along the axis cylinders; it seems to act as an irritant to the motor cells in the anterior horn of the spinal cord and also interferes with the normal inhibition of motor impulses exercised by the upper motor neuron over the lower, producing increased muscular tonus and tonic spasms. This affords an explanation of local tetanus. Another view is that in cases of general tetanus the toxin is absorbed by the lymphatics and reaches the central nervous system by the blood-stream, while local tetanus is supposed to be due to a direct action of the toxin on the nerve-endings in muscles.

For the prophylaxis of tetanus, *formol-toxoid* (*cf.* diphtheria toxoid) is now being used to produce active immunity among troops on active service. Two doses of 1 c.c. each are administered intramuscularly or subcutaneously, at an interval of 6 to 8 weeks.

Antitoxin.—An antitoxic serum can be obtained by immunising horses with toxoid and toxin. This serum is of great value in the prophylaxis of tetanus. Its use as a curative agent after the development of tetanus is less reliable than, and not nearly as effective as, the corresponding antitoxin treatment in diphtheria.

Tetanus antitoxin is now standardised in Great Britain in terms of the "International Unit" (I.U.) which was originally defined as five times the quantity of antitoxic serum required to preserve the life of a 350-grams guinea-pig for ninety-six hours when mixed with 100 M.L.D. of toxin and injected subcutaneously. The method of determining the potency of a serum is similar to that used in assaying diphtheria antitoxin, *i.e.* by comparison with a preserved standard antitoxic serum. For prophylactic purposes 3000 I.U.'s should be given subcutaneously and repeated on one or two occasions at weekly intervals if considered necessary, *e.g.* wounds with severe sepsis or necrosis of tissue. In the treatment of a case the antitoxin has usually been administered both intraspinally (40,000 to 60,000 I.U.'s) and intravenously (20,000 I.U.'s), and the administration is repeated if necessary. Reliance is now generally placed on the administration of a large dose intravenously (*e.g.* 200,000 I.U.'s) without intraspinal injection, subsequent intramuscular injections being given if necessary.

Occurrence.—The spores are widespread in nature, but are specially prevalent in manure and manured soil. Tetanus bacilli may occur naturally in the intestine of certain animals—*e.g.* horses, cattle, sheep—and have been noted occasionally in the human intestinal contents. It is uncertain whether this organism flourishes as a saprophyte in nature, or is derived entirely from an animal source.

Tetanus is usually the result of a wound contaminated with *B. tetani* spores. The infection remains strictly localised, and the tetanic condition is due to the diffusible toxin absorbed in the central nervous system.

Certain conditions favour the germination of the spores and the propagation of the organisms in the tissues—*e.g.* infection by other organisms (*B. welchii*, pyogenic cocci, etc.), foreign bodies such as pieces of clothing carried into the wound, necrotic tissue and effused blood. It has been shown that the spores germinate only under a reduced oxygen tension.

B. tetani infection may occur in the uterus, as in cases of septic abortion, and also in the umbilical wound of new-born infants. Cases of post-operative tetanus have been recorded, due to imperfectly sterilised catgut or dressings.

DIAGNOSIS

Films may be made from the wound exudate and stained by Gram's method, but the appearance of "drum-stick" bacilli is not conclusive evidence of the presence of *B. tetani*, as other organisms having round terminal spores, which are practically identical in morphological characters, may be present (*vide* p. 511). Moreover it is often difficult or impossible to detect the tetanus bacilli in wounds by microscopic examination.

The more reliable method for diagnostic purposes is to produce tetanus in white mice by subcutaneous injection of a filtrate of an anaerobic fluid culture from the wound. A control test should be included in which tetanus antitoxin has been administered as a prophylactic (*cf.* diphtheria virulence test, p. 380). While significant results may sometimes be obtained with impure or mixed cultures from the wound it is essential that the tetanus bacillus if possible should be obtained in pure culture so that it can be identified by its biological characters and its specific toxicity. In isolating the organism advantage is taken of the tendency of *B. tetani* colonies to spread and extend beyond the growth of other bacteria. In Fildes's method the material is incubated anaerobically in 5 per cent. peptic-blood-broth[1] for two to four days at 37° C.. The culture is then heated at 65° C. for half-an-hour to kill spreading non-sporing organisms such as *B. proteus*. The condensation water of a peptic-blood-agar slope is inoculated from the heated culture, and incubated at 37° C. under anaerobic conditions. After twenty-four to forty-eight hours the edge of the culture is examined with a low-power binocular microscope or hand-lens, when a growth of tetanus bacilli is seen as a mass of very fine filaments. Subcultures from the marginal growth usually yield pure cultures of *B. tetani*. (It is advantageous to keep the peptic-blood-agar tubes until the surface of the medium is dry at the top.)

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[1] A mixture of 150 c.c. saline solution, 6 c.c. pure HCl, 50 c.c. defibrinated sheep blood and 1 gram of pepsin (B.P. granulated) is heated at 55° C. in a stoppered bottle for two to twenty-four hours. There is then added sufficient 20 per cent. NaOH solution (usually about 12 c.c.) until a sample of the mixture diluted with water gives a permanganate-red colour with cresol-red indicator. Pure HCl is now added drop by drop until a sample of the mixture shows almost no change of colour with cresol-red, but a definite red tint with phenol-red. It is important to avoid excess of acid. Chloroform, 0·25 per cent., is added and the mixture shaken vigorously. This peptic digest of blood keeps well for months. For use it is heated to 55° C. for thirty minutes to remove the chloroform, and added to broth or agar in the proportion of 2 to 5 per cent.

OTHER ANAEROBIC BACILLI OF INFECTED WOUNDS

These organisms, belonging to the genus *Clostridium*, are associated with rapidly spreading inflammatory oedema, necrosis and gangrene of the tissues, and gas production, occurring as a complication of wound infection. They are all sporing organisms and their source is animal and human excreta. They were responsible for the gas-gangrene which was so prevalent among the armies in Europe during the war of 1914–1918.

The infection usually results from the contamination of a wound with soil (particularly that of manured and cultivated land), dirty clothing, street dust, etc., but may also be derived in some cases from the skin especially in areas of the body that may be contaminated with intestinal organisms.

In nature, these organisms play an essential part in the process of putrefaction.

They have been broadly classified into two types: (1) *saccharolytic*, (2) *proteolytic*.

(1) The saccharolytic organisms are characterised by their rapid and vigorous growth in carbohydrate media with the production of acid and abundant gas. If grown in a medium containing both carbohydrate

and protein—*e.g.* meat medium (*vide* p. 153)—there is rapid production of acid and gas, but *no digestion* of the meat. The cultures may have a slightly sour smell, and the meat is often reddened by the acid.

(2) The proteolytic type is characterised by the digestion of protein. In meat medium the proteolytic ferments of these organisms decompose and blacken the meat with the formation of foul-smelling sulphur compounds. The protein is also broken up into amino-acids, and small white feathery masses of tyrosine crystals may be seen in cultures of certain species.

The more important organisms exemplifying these types are the following :—

Saccharolytic	*Proteolytic*
B. welchii	*B. sporogenes*
Vibrion septique	*B. histolyticus*
B. oedematiens (*B. novyi*)	*B. tetani*—slightly proteolytic (*vide* p. 505)
B. tertius	
B. fallax	

There is, however, no hard and fast line of demarcation between the two groups. Thus, strains of *B. welchii* produce small amounts of amino-acid, and *B. sporogenes*, though essentially proteolytic, has saccharolytic properties.

For further detailed information on the anaerobes of infected wounds, the *Medical Research Committee's Special Report*, No. 39, 1919, should be consulted. A Table (quoted from p. 47 of the Report) is given on p. 511, showing the general classification of these organisms. See also **Appendix**, and *Medical Research Council's War Memorandum*, No. 2, Revised Second Edition, 1943.

It must be emphasised that the separation and cultivation of these anaerobes is much more difficult than in the case of the aerobes. In wounds there is practically always a mixed infection, so that the isolation of the various bacteria must be undertaken. Simple plating alone is frequently not sufficient as in the case of the aerobes, but alternate growths on plates and in fluid media has sometimes been found necessary before

TABLE SHOWING CLASSIFICATION OF SPORING ANAEROBES

	Both proteolytic and saccharolytic properties		*Slight proteolytic but no saccharolytic properties*	*Saccharolytic but no proteolytic properties*	*Neither saccharolytic nor proteolytic properties*
	Proteolytic properties predominating Coagulated serum and gelatin are liquefied	Saccharolytic properties predominating Serum not liquefied; gelatin liquefied	Serum not liquefied; gelatin liquefied	Neither serum nor gelatin liquefied	Neither serum nor gelatin liquefied
Central or subterminal spore .	*B. sporogenes* *B. parasporogenes* *B. histolyticus* *B. aerofetidus* *B. bifermentans*	*B. welchii* *Vibrion septique* *B. chauvoei* *B. oedematiens* (*B. novyi*)		*B. fallax* *B. multifermentans tenalbus*	
Oval terminal spore . .				*B. tertius*	*B. cochlearius*
Spherical terminal spore .			*B. tetani*	*B. tetanomorphus* *B. sphenoides*	

This Table is quoted by permission of the Controller of H.M. Stationery Office, from the *Medical Research Committee's Special Report*, No. 39.

a pure culture is obtained. Much of the confusion in the early literature has been due to the failure to separate mixed cultures, and according as the medium contained carbohydrate or protein, so the saccharolytic or proteolytic organisms in the mixtures predominated.

In the **Appendix** an account is given of the methods at present in general use for the bacteriological examination of wounds, and this includes an outline of the procedures for isolating and identifying the more important anaerobes with particular reference to cases of gas-gangrene.

For infection by these anaerobes to take place, *dead tissue or blood clot must be present.* The organisms cannot attack living tissue directly. Thus, in war casualties infection tends to occur where there are deep lacerated wounds caused by irregularly shaped pieces of shell, and into which muddy clothing and particles of earth were carried. Spores are then introduced under most favourable conditions for the development of the organism.

In the contaminated wound the saccharolytic organisms are the first to grow and may spread rapidly in the interstitial substance of lacerated muscles. Toxic products of growth gain access to the muscle and cause death of the fibres. The saccharolytic organisms (usually *B. welchii*) may actually multiply within the sarcolemma sheath, fermenting the muscle sugar and forming abundant gas. The death of the muscle is assisted by the oedema and gas formation, which tend to cut off the blood supply. The process may spread throughout the length of the affected muscles. There is also marked toxaemia. The proteolytic organisms (usually *B. sporogenes*) follow later, digesting the dead muscle and causing blackening and the foul odour.

Gas-gangrene may also be located in subcutaneous tissue, *e.g.* where there has been extravasation of blood. Less severe forms of anaerobe infection may occur without the typical toxaemia ; such wounds have a foul odour and gas bubbles form in them. Moreover it must be remembered that potentially pathogenic anaerobes may be cultivated from a wound which never shows any signs of gas-gangrene.

BACILLUS WELCHII (*Clostridium welchii*, B. Perfringens, B. Aerogenes Capsulatus)

The most frequent organism and commonest cause of gas-gangrene.

Morphology.—A relatively large Gram-positive bacillus, about 4–6μ by 1μ, with square or rounded ends, occurring singly or in pairs, and often capsulated when seen in the tissues. In sugar media the bacilli are shorter, while in protein media they tend to become filamentous. The bacilli are non-motile. Spores are formed, but only in the absence of fermentable carbohydrates. They are oval and subterminal.

Culture.—Anaerobe. Optimum temperature about 37° C.. Grows best on carbohydrate-containing media —*e.g.* glucose-agar.

Surface colonies—large, round, smooth, regular, opaque disks. (Another type of colony is also observed, with an opaque centre and a transparent border which is radially striated.) On blood-agar the colonies are haemolytic.

In milk—acid, clot and gas production result; the gas breaks up the clot, producing the characteristic " stormy clot " reaction; the culture has a sour, butyric-acid odour.

Gelatin is liquefied.

Coagulated serum—no liquefaction usually occurs; some strains, *e.g.* from animal diseases (*vide infra*), have been stated to liquefy serum.

B. welchii is actively saccharolytic and ferments with gas production, glucose, lactose, saccharose, maltose, starch, and, in the case of some strains, glycerol and inulin. Mannitol, dulcitol and salicin are not fermented.

In meat medium (*vide* p. 154)—the meat is reddened *and no digestion occurs.*

Filtrates of cultures are strongly haemolytic, and a lethal exotoxin can be demonstrated by intravenous injection of mice. A local necrotising action on muscle tissue is also a property of toxic filtrates.

An antitoxic serum for prophylactic and therapeutic use is obtained by immunising horses with the toxin (*vide* p. 33).

Occurrence.—Apart from its pathological relationships, *B. welchii* occurs normally in the large intestine of man and animals. It may invade the blood *ante mortem*, and multiplying in internal organs after death produces the small gas cavities sometimes noted (*e.g.* in the liver) at *post mortem* examinations. Apart from wound infections it may occur in uterine infections (*e.g.* septic abortion) and in infections of the intestinal tract, gall bladder and the urinary system.

In certain conditions the numbers present in the bowel are greatly increased—*e.g.* pernicious anaemia, intestinal obstruction. In pernicious anaemia the associated achlorhydria is probably a factor which allows this organism to flourish, and in this condition *B. welchii* may be found even in the duodenum and stomach.

B. welchii may also occur in gas-oedema of the muscles in cattle and sheep.

Animal Inoculation.—The virulence varies greatly with different strains. Some are markedly pathogenic to guinea-pigs by subcutaneous injection of culture and the animal may die within twenty-four hours. At autopsy a spreading inflammatory oedema with gas production is noted in the subcutaneous tissue, and necrosis in the underlying muscles which are sodden, friable and pink. Organisms from cultures washed with saline solution to free them from toxin and other soluble products are practically non-pathogenic. Apparently the products of growth of the bacillus increase its aggressiveness.

Pigeons are the most susceptible of laboratory animals to experimental inoculation.

Lamb dysentery.—This condition has been shown to be due to a type of *B. welchii*, *viz.* Type B. (The classical type occurring normally in the intestine of man and animals is designated Type A.) Its pathogenicity depends on exotoxins, for which an antitoxic serum can be obtained. This serum has been utilised with great success in the prophylaxis of the disease, the lambs being injected as soon after birth as possible. Ewes may also be immunised (during pregnancy) with toxoid or with a formolised culture. It is supposed that the antibodies produced by such immunisation are conveyed to the lamb in the colostrum.

Another type of *B. welchii*, named *B. paludis* (or Type C), has been found associated with a disease of sheep known as "struck," the condition being essentially a toxaemia due to absorption of the bacterial toxins from the small intestine. A similar disease has been reported in Australia, and designated "infectious entero-toxaemia"; the associated organism, a further type of *B. welchii*, has been designated *B. ovitoxicus* (or Type D). A disease affecting lambs in Australia and in this country, often called "pulpy kidney disease," is of similar nature and aetiology to infectious entero-toxaemia. Appropriate methods of immunisation may be carried out against these diseases, as in the case of lamb dysentery.

Five different toxic factors, distinguishable by neutralisation tests with antitoxic sera, have been demonstrated among the organisms of the *B. welchii* group, and individual strains may produce more than one of these. The neutralisation tests are made by the intravenous injection of mice with mixtures of toxin and antitoxin. The types of *B. welchii* referred to above differ as regards their production of the several toxins. The differences are indicated in the following table (according to Dalling and Ross):—

Type	*Toxic Factors*					*Antitoxin for Type*
	α	β	γ	δ	ε	
A (classical *B. welchii*)	+	−	−	−	−	A neutralises only type A toxin.
B (Lamb dysentery bacillus)	+	+	+	±	+	B neutralises types A, B, C and D toxins.
C (*B. paludis*)	+	+	+	+	−	C neutralises types A and C toxins.
D (*B. ovitoxicus*)	+	−	−	−	+	D neutralises types A and D toxins.

+ toxic factor produced, − not produced, ± not invariably produced.

Types B and D are weakly haemolytic as compared with A and C. Strains of type D may undergo transformation serologically to type A through loss of their specific toxic factor.

VIBRION SEPTIQUE (*Clostridium septicum*)

Morphology and Staining.—Moderately large bacillus, with rounded ends, about 3–10μ by 0·6–1μ. Motile, with peritrichous flagella. Tends to grow also in the form of long curved filaments. In the tissues it develops into large, swollen, Gram-positive, lemon-shaped forms, which are designated "citron bodies." Spores are readily formed and are oval, central or subterminal, and "bulging." *V. septique* stains Gram-positively as a rule, but degenerate forms are Gram-negative.

Culture.—Anaerobe. Optimum temperature, 37° C.. Capable of growing on ordinary media. Glucose promotes growth.

Agar stab—a white line of growth with short lateral processes.

Surface colonies—irregular, transparent, droplet-like colonies, later becoming greyish and opaque, with projecting radiations somewhat like those of *B. tetani.*

Milk—slight acid is formed, and the milk may be slowly clotted but often there is little change.

Gelatin is liquefied.

Coagulated serum—no liquefaction.

Meat medium—meat is reddened but not digested.

Various sugars are fermented, *e.g.* glucose, lactose, maltose and salicin, but not mannitol or saccharose.

Exotoxin can be demonstrated in cultures and a specific antitoxin can be obtained by immunising animals.

Animal Inoculation.—Subcutaneous injection of cultures in laboratory animals produces a spreading inflammatory oedema, with slight gas formation in the tissue. The organisms invade the blood and the animal dies within a day or two. Smears from the liver show long filamentous forms and also "citron bodies."

V. septique is responsible for *Braxy* in sheep and some cases of *Black-leg* in cattle and sheep (*vide infra*).

A vaccine consisting of formolised culture of this organism has been used with success in the prevention of braxy.

BACILLUS SPOROGENES (*Clostridium sporogenes*)

An anaerobic motile bacillus, with peritrichous flagella, and oval, central or subterminal spores. It is about the same size as *B. welchii*, but more slender. It is typically Gram-positive in young cultures, but Gram-negative forms are frequent in older cultures.

A stab culture shows a growth like that of *B. tetani*, with lateral radiations or spikes. Surface colonies present a medusa-head appearance (*cf. B. anthracis*) if the plate is dry but may be irregular and feathery if moisture is present. Cultures have an exceedingly putrid odour. In milk the casein is precipitated and digested. In meat medium the meat is blackened and digested. Coagulated serum is liquefied. Glucose and maltose are fermented, with gas production.

The organism decomposes protein, producing amino-acids, ammonia, sulphuretted hydrogen, etc..

It is non-pathogenic to laboratory animals.

Bacillus oedematiens (*Clostridium novyi*).—This organism resembles *B. welchii* in morphology but is somewhat larger and more pleomorphic. It possesses peritrichous flagella but its motility is not active. The spores are central or subterminal. Surface colonies are transparent, flat and tend to fuse, forming a spreading film of growth. Deep colonies are small, irregular, "woolly" or "snow-flake"-like balls of growth. In milk, acid is produced; late clotting may occur. Gelatin is liquefied; coagulated serum is not digested. The organism is actively saccharolytic and various sugars are fermented. In cooked-meat medium the meat is reddened but not digested. A highly potent exotoxin is produced and this organism has been found specially associated with a "toxic" form of gas-gangrene.

B. oedematiens causes "Black disease" among sheep in Australia and New Zealand. This condition is activated apparently by the invasion of the liver fluke. A necrotic hepatitis is produced followed by toxaemia and death. Formolised culture may be used as a prophylactic vaccine.

Bacillus fallax (*Clostridium fallax*) resembles *B. welchii* in some respects, and has sometimes been mistaken for it (hence the name "*fallax*"). It is, however, shorter and more slender. The spores are usually subterminal. In milk the organism produces clotting and gas formation, but these changes take place slowly (as compared with *B. welchii*). It does not liquefy either gelatin or coagulated serum, and is non-proteolytic. It possesses saccharolytic properties. An exotoxin is formed, and the organism is pathogenic on experimental inoculation in animals.

Bacillus tertius (*Clostridium tertium*).—In morphology this organism tends to be long and slender. It is weakly motile. The spores are terminal, and when fully developed oval in shape. It is not a strict anaerobe. Neither gelatin nor coagulated serum is liquefied, but the organism shows active saccharolytic properties. In milk acid is formed with a certain amount of clotting and gas production. Meat is reddened but not digested. Its pathogenicity is doubtful, but when present in wounds it may give rise to gas production. No exotoxin is produced.

Bacillus histolyticus (*Clostridium histolyticum*) resembles *B. sporogenes* and is actively proteolytic. It is not a strict anaerobe. In meat medium digestion occurs with the formation of white crystalline masses consisting of tyrosine. When cultures are injected into animals, *in vivo* digestion of the tissues results. This organism also produces an exotoxin analogous to that of *B. welchii*, etc..

DIAGNOSIS (see also Appendix)

In a case of gas-gangrene film preparations are made from the exudate which may be taken with a swab or a capillary pipette. The films are stained by Gram's method. Some indication of the morphological type or types of anaerobe present may be obtained in this way, preparatory to cultivation.

Presumptive evidence of the presence of *B. welchii* may be obtained by inoculating melted glucose-agar in tubes at 45° C. and allowing it to solidify. After twelve hours' incubation the medium is split up by abundant gas, and the cotton-wool stopper and even some of the medium may sometimes be forced out of the tube. Microscopical examination shows the typical Gram-positive bacilli. Inoculation into milk, which is incubated under anaerobic conditions, often shows the typical "stormy clot" in six to twelve hours.

Primary cultivation in milk may also be carried out to obtain presumptive evidence of the presence of *B. welchii*; if the milk is contained in tubes and boiled previously to expel air and then after cooling heavily inoculated and covered with melted vaseline, it is not necessary to incubate the tubes in an oxygen-free atmosphere.

This reaction, however, is not given by all strains, and an alternative test has been used for the early recognition of *B. welchii*, *viz.* Nagler's reaction, which consists in the production of turbidity and a fine "curd" in human serum by the toxin of the organism.[1]

The presence of *B. sporogenes* or other proteolytic type can be detected by inoculating the material into meat medium. After two days' incubation, blackening and digestion of the medium with a foul odour is presumptive evidence of the presence of these organisms. On microscopic examination, the bacilli with central or subterminal spores are seen.

The exact identification of the anaerobes involves in the first place most careful separation of pure cultures. An initial culture may be made in meat medium and from this a subculture is obtained on a blood-agar plate incubated anaerobically; a blood-agar plate should also be inoculated directly. Isolated colonies of Gram-positive bacilli are subcultured in meat medium and the derivative cultures are re-plated. From these plates isolated colonies are again subcultured. To ensure purity of the ultimate growths it is advisable to repeat the platings two or three times, on each occasion subculturing isolated colonies first in meat medium. The characters of the pure cultures can then be studied in detail and their identity established—the features of the more important sporing anaerobes have been given above, and are also tabulated in the Appendix.

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[1] See **Appendix**, and Hayward, N. J., *Brit. Med. J.*, 1941, i, 811.

Gas-gangrene Antisera.—A polyvalent serum is available for prophylactic use and for treatment of cases in which the causal organism has not been determined. The prophylactic dose, given intramuscularly (*or in urgent cases intravenously*). is 9000 international units *B. welchii* antitoxin, 4500 units *V. septique* antitoxin and 3000 units *B. oedematiens* antitoxin. The therapeutic dose, given intravenously, should be at least three times the prophylactic dose, and the administration should be repeated as necessary. Monovalent sera are also available for the treatment of cases after the causal organism has been identified.

Chemotherapy.—The sulphonamide compounds have been used both in the prophylaxis and treatment of gas-gangrene; they may be administered orally and as prophylactics have also been applied locally in powdered form. The effect varies with the different organisms and is most evident in the case of *B. welchii.*

Local application of penicillin has also been employed as a prophylactic measure; systemic administration is required for the established infection.

BACILLUS BOTULINUS (*Clostridium botulinum*)

The organism of "Botulism," a fatal form of food-poisoning characterised by pronounced toxic effects mainly on the parasympathetic system—*e.g.* oculomotor paralysis, pharyngeal paralysis, aphonia, etc.. Animals are also subject to this disease, *e.g.* forage poisoning of horses, "Lamziekte" of cattle in South Africa, "Limber-neck" of fowls. Three main types of *B. botulinus* have been differentiated and designated A, B and C.

Morphology and Staining.—A sporing bacillus with rounded ends, about 4–6μ by 0·9–1·2μ, occurring singly and in pairs. Spores are oval, subterminal and slightly "bulging." The bacilli are motile, with peritrichous flagella, and stain Gram-positively unless degenerate.

Culture.—Anaerobe. The optimum temperature has been variously stated; earlier workers found growth occurred best at 20°–30° C.; more recent observations indicate that the optimum is about 35° C.. Grows on ordinary media; meat medium yields abundant growths.

Agar-stab culture—a white line of growth, stopping short of the surface, with short lateral spikes or radiations; gas production is marked, especially in glucose-agar.

Surface colonies—large, greyish, irregular, semi-transparent, with a central "nucleus" and a reticular border. Cultures emit a rancid, butyric-acid odour.

Gelatin is liquefied.

Coagulated serum—types A and B produce slow liquefaction; type C, however, does not liquefy serum.

Milk—types A and B precipitate and digest the casein; type C is inactive in this respect.

Ferments glucose and maltose—type A also ferments salicin and glycerol, type B ferments glycerol but not salicin, type C does not act on either of the substances.

Cooked meat—types A and B digest and blacken the meat; type C does not digest meat.

In culture media and in contaminated foods, *B. botulinus* produces a powerful exotoxin which is responsible for the pathogenic effects in the disease. This toxin is destroyed when exposed to a temperature of 90° C. for forty minutes.

The different types of the bacillus produce toxins which are immunologically different and neutralisable only by the appropriate antitoxin; thus, antitoxin produced from toxin A does not neutralise toxin B, and vice versa.

Types A and B are those associated with botulism in the human subject.

All types have been found in animal botulism, but in horses, cattle and fowls mainly type C. Carrion flies and their larvae may harbour *B. botulinus* and these may be a source of botulism in poultry. It may be noted that further types (D and E) have been described in animal botulism but there has been some confusion between types C and D. These additional types still require clearer definition.

Occurrence.—Botulism has been found to originate from a considerable variety of preserved foods—*e.g.* ham, sausage, canned meats and vegetables, etc..

It is due, not to the formation of toxin by the organism in the intestine, but to the absorption from the stomach and upper duodenum of *toxin pre-formed by the bacillus growing in the food.*

Canned foods responsible for botulism frequently exhibit signs of spoilage.

B. botulinus is a saprophytic organism and is widely distributed. Its natural habitat is soil, even virgin and forest soil. It may be found in vegetables, fruits, leaves, mouldy hay, ensilage and animal manure.

The spores of *B. botulinus* withstand moist heat at 100° C. for several hours. They are destroyed at 120° C. (moist heat) usually within five minutes. Insufficient heating in the process of canning foods is an important factor in the causation of this form of poisoning.

In cases of botulism the bacillus may be demonstrated in the stomach contents and faeces, and *post mortem* in the intestinal contents and in the liver and spleen.

It can also be isolated from the food responsible for the outbreak.

Animal Inoculation.—Laboratory animals are susceptible to experimental inoculation and feeding with cultures. The resulting condition resembles in its symptomatology the human disease; at autopsy marked congestion of the internal organs, extensive thrombosis and haemorrhages are noted.

Antitoxin can be prepared by immunising animals with toxin preparations, and is used therapeutically. A bi-valent serum containing antitoxins to both the A and B types of toxin should be employed.

DIAGNOSIS

As the condition of botulism is essentially a food intoxication, the suspected food calls for investigation. It is macerated in sterile salt solution, heated at 65° C. for half-an-hour to eliminate non-sporing bacteria, and then cultures are made under anaerobic conditions—*e.g.* in meat medium. A culture-filtrate is obtained and its toxicity tested by injection of guinea-pigs or mice. For control purposes the filtrate is also injected into animals along with types A and B antitoxin respectively. *B. botulinus* can be isolated in pure culture by appropriate plating methods, and identified by its biological characters and its toxicity.

An extract should also be made from the food, sterilised by filtration through a porcelain filter, and injected subcutaneously into guinea-pigs.

BACILLUS CHAUVOEI (*Clostridium chauvoei*)

The causative organism of most cases of quarter evil (black-leg, or symptomatic anthrax) in cattle and sheep, a disease characterised by a swollen and emphysematous condition of the subcutaneous tissues and muscles. The infection frequently affects the fore- and hind-quarters, which become dark or almost black in colour.

Morphology.—Resembles closely the *Vibrion septique* and is 3–5μ in length and 0·5–0·6μ broad. Individual organisms tend to occur singly or in pairs and not in long filaments. They are motile, having numerous peritrichous flagella. Spores are usually central or subterminal in position, elliptical in shape, and are broader than the bacillus.

Staining.—Gram-positive in young cultures but older forms may be Gram-negative. Stains readily with ordinary dyes.

Culture.—Anaerobe; optimum temperature 37° C., but grows at room temperature. Grows on ordinary medium but a blood or meat medium is preferable.

Colonies on agar—greyish-white, transparent, irregular, with radiating filamentous processes.

Stab culture in glucose-agar—growth only commences some distance below the surface ; along the puncture a whitish line appears with lateral projections, the growth being luxuriant.

Milk—unchanged or may occasionally show acid formation with partial clotting.

Gelatin is liquefied.

Coagulated serum is not liquefied.

Meat medium—meat is reddened and is not digested.

Ferments glucose, lactose, saccharose, but not mannitol, salicin or inulin.

A lethal exotoxin is obtained in glucose-broth cultures especially if calcium carbonate be added to neutralise the acid produced. Culture-filtrates are also haemolytic.

Occurrence and Pathogenicity.—The disease occurs in cattle and sheep. The organism is pathogenic for guinea-pigs and mice, these animals dying twenty-four to thirty-six hours after inoculation. At autopsy there is an extensive blood-stained oedema round the site of inoculation and the muscles present a dark red or black appearance, while there is a considerable amount of gas produced ; the bacillus is present in the heart blood in pure culture. It has been supposed that the natural disease results from infection of a wound by the spores which may be present in the soil of infected pastures.

Immunity.—Prophylactic immunity against the disease has been practised by the inoculation of a dried powder of the muscles of animals dead of the natural disease, the powder being subjected to a suitable temperature to ensure the attenuation of the virulence of the contained spores. A mixture of this preparation with antitoxin has also been used to protect susceptible animals. Aggressin (*vide* p. 33) has likewise been used for immunisation. More recently a formolised culture of *B. chauvoei* in broth has been applied with successful results. An antitoxic serum has been used for therapeutic purposes.

V. septique may also be responsible for a condition similar to " black-leg."

In the *differentiation of V. septique and B. chauvoei*, stress has been laid on the morphological elements seen in infected guinea-pigs : *B. chauvoei* exhibits " citron " and club-shaped forms, but no elongated filaments are observed on the peritoneal surface of the liver of inoculated animals, as in the case of *V. septique*. *B. chauvoei* ferments saccharose but not salicin ; *V. septique* ferments salicin but not saccharose. The two organisms, however, are closely related.

CHAPTER XIX

ACTINOMYCES; LEPTOTHRIX; BACILLUS RHUSIOPATHIAE (SWINE ERYSIPELAS); THE ORGANISM OF BOVINE PLEURO-PNEUMONIA; BACILLUS FUSIFORMIS; BACILLUS NECROPHORUS

ACTINOMYCES

The causative organism of actinomycosis in animals and man.

This organism belongs to the genus which in the older classification and nomenclature was called *Streptothrix* and was placed among the Higher Bacteria (*vide* p. 5). The term *Actinomyces*, at one time used as a specific name, is now applied in a generic sense (*vide* p. 17).

Morphology.—It grows as a mycelium or felted mass of branching filaments which are comparatively slender ($0{\cdot}8\mu$–1μ thick). In the centre of this mycelial colony the filaments interlace irregularly, but at the periphery there is a tendency to radial arrangement. The organism shows true dichotomous branching. The mycelium is embedded in a groundwork or matrix. In old growths the filaments become matted together into a structureless mass. They also show fragmentation into bacillary and coccal forms. The formation of conidia has not been demonstrated. In culture the typical mycelium may not be so obvious as in the tissues, and the growth may be composed largely of shorter bacillary forms resembling a diphtheroid bacillus; among these, however, are seen some longer branching filaments which reveal the true character of the organism.

Ørskov's method (*vide* p. 188) is a very suitable technique for studying the morphology of the organism in culture.

Growing in the tissues (especially in animals) the actinomyces colony develops pyriform or club-shaped structures at the periphery, supposed to result from the swelling of the sheath at the extremities of peripheral radial filaments. These "clubs" lie radially with their wide end outwards and as seen in tissue sections form a complete ring round the colony. In animal lesions the clubs may constitute the main morphological feature of the older colonies, owing to the degeneration of the filaments which become fused into a structureless mass in the centre of the colony. The clubs have sometimes been regarded as a defensive mechanism on the part of the organism against the tissues of the host. In human lesions "club" formation is less frequent than in animals.

Staining.—The filaments are Gram-positive. The clubs usually stain Gram-negatively, but are acid-fast, and can be stained differentially by the Ziehl-Neelsen method, 1 per cent. being substituted for 20 per cent. sulphuric acid.

Culture.—Two main cultural types have been recognised :

(1) The micro-aerophilic type, first described by Israel and Wolff, which is the prevalent form in animal and human lesions.

(2) The aerobic type, exemplified by Boström's classical strain ; it has been suggested that this type is not pathogenic and that its occurrence in lesions represents a secondary contamination ; similar organisms are common saprophytes in soil, on grain and grasses, and may be found in the mouth and alimentary tract of animals and also man.

Micro-aerophile Type (*Actinomyces bovis*).—The optimum temperature is 37° C., and growth does not occur at temperatures much below the optimum. The organism requires a reduced oxygen tension or increased carbon dioxide pressure for its growth on culture medium. Blood-agar is a suitable medium for routine cultivation and growth can be obtained conveniently by the ordinary anaerobic methods.

On agar, colonies are raised and nodular, cream-coloured, opaque disks which show a rosette form or an irregular outline and are firmly adherent to the medium.

Strains from animals may yield softer and smoother colonies with a more regular outline. Animal strains may also be more oxygen-tolerant than human strains.

A shake culture in a tube of agar presents a characteristic distribution of the colonies, which are most numerous in a zone about 10–20 mm. below the surface —*i.e.* where there is only a trace of free oxygen present or an optimal concentration of carbon dioxide.

If the organism is grown in the form of a stab culture in agar, growth is also at an optimum in a similar zone.

It should be noted that the cultural appearances of this type of organism are subject to variation, and varying conditions of cultivation may be responsible for variability in cultural characters. Dissociation may also occur in culture and give rise to variants which differ from the parent strain.

As compared with the aerobic actinomyces, this type is less active chemically: it is non-proteolytic, non-haemolytic and does not produce pigment. Saccharolytic action, however, can be demonstrated, various sugars being fermented (without gas).

By experimental inoculation of cattle and laboratory animals (*e.g.* rabbits, guinea-pigs) circumscribed nodular or "tumour-like" granulomatous lesions have been produced, in which colonies of the organism are demonstrable. Laboratory animals, however, are not readily infected with this organism by experimental inoculation. Successful results are most likely to be obtained by intraperitoneal injection of rabbits.

Serological grouping.—Strains of bovine origin form apparently a separate group from the human strains.

For further information regarding these organisms reference should be made to Erikson, D., *Med. Res. Council Spec. Rep. Ser.* No. 240, 1940.

Aerobic Type.—The aerobic mycelial organisms described even in typical cases of actinomycosis have been somewhat heterogeneous in biological characters and probably represent different species.[1] Only one of these will be described here, *viz.* the classical actinomyces of Boström.

Temperature range—20°–40° C., optimum—37° C.. Grows on ordinary media, but the presence of serum or glycerol encourages growth.

On agar—grows slowly; colonies begin to appear after four or five days; when well developed, they stand out on the surface of the medium as discrete, rounded, yellow, transparent knobs, often likened to "amber drops"; they are firmly adherent to the medium; older colonies become umbilicated and assume a dry "powdered" appearance due to the development of aerial mycelium.

In gelatin—slow liquefaction occurs.

No pathogenic effects have been demonstrated by experimental inoculation.

Occurrence.—Actinomycosis is an infective granuloma, occurring mainly in cattle, sheep and pigs, and occasionally in man. In human cases the lesions usually show a suppurative tendency, and the pus contains colonies of the parasite in the form of small round granules about the size of a pin-head, which are occasionally of a bright yellow colour (like grains of sulphur). These granules can be recognised by the naked eye if the pus is examined in the form of a thin layer on a slide. The commonest avenue of infection in man is through the mucosa of the mouth or throat. Not infrequently the infection starts in connection with a carious tooth or in the tonsil. The initial infectivity of the organism is probably weak, and invasion of the tissues may occur only in the presence of some additional factor. Primary foci have been noted in animals, and occasionally in man, around fragments of grain embedded in the mucous membrane of the mouth. It was at one time thought that grain was a primary source of the infection, but it is likely in these cases that the parasite has gained independent access to the body, and that the grain fragment facilitates the establishment of the organism in the tissue. The prevalent type of actinomyces (*vide supra*) is, in fact, a strict parasite, and incapable of a saprophytic existence on grain. Where the avenue of infection is by the mouth or throat, the primary lesions involve the soft tissues of the mouth and neck, the periosteum of the jaw, and even the vertebrae. In some cases the avenue of infection may be through the mucosa of the bowel (*e.g.* caecum), or the primary lesions may be in the lung. There is a considerable amount of evidence that the actinomyces sometimes occurs as a commensal in the mouth, throat (*e.g.* crypts of tonsils) and the alimentary tract, and that actinomycotic infection is endogenous.

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[1] See Erikson, D., *Med. Res. Council Spec. Rep. Ser.* No. 203, 1935.

Metastatic lesions are also liable to occur (*e.g.* in the liver, brain, kidney or lung) by blood-stream spread.

The organism is found in tissue lesions, as in pus, in the form of compact colonies or granules which are visible even to the naked eye, and these present the microscopic appearances described above, varying according to the age of the individual colonies.

BACILLUS ACTINOMYCETEM COMITANS.—Besides the mycelial organism, a small Gram-negative cocco-bacillus (morphologically resembling the *Brucella* group) is frequently present in large numbers in the actinomyces colony. This organism can be cultivated independently. Growth occurs under aerobic conditions, and consists of small colonies somewhat like those of a streptococcus and entirely different from the actinomyces. Various carbohydrates are fermented with slow acid production.

This organism has been regarded as a distinct species, and has been designated *B. actinomycetem comitans*, but its actual relationship to the actinomyces is unknown. Experimental inoculation does not produce any specific lesions.

DIAGNOSIS

If the pus from an actinomycotic lesion is spread out in a thin layer in a Petri dish or on a microscope slide, the characteristic colonies or granules can be recognised with the naked eye. For microscopic examination the granules in a drop of pus are " crushed " between two slides. In this way films can be prepared and then stained by Gram's method.

The granules can easily be separated by shaking up the pus with water in a test-tube, allowing them to sediment, and collecting them in a capillary pipette. They are then deposited on a slide and films made by crushing. Preparations obtained in this way are more satisfactory than those made directly from pus in which the granules may be relatively scanty.

Microscopic demonstration of Gram-positive branching filaments arranged in the form of mycelium is generally sufficient for clinical diagnosis.

In tissue lesions the colonies can be recognised by preparing histological sections and staining by Gram's method, and, in the case of animal lesions, by the modified Ziehl-Neelsen method described above.

To cultivate the organism it is essential that actual granules should be used for inoculating the medium. For this purpose, the pus is mixed with sterile water, the granules are allowed to sediment or deposited by centrifuging, and then removed with a pipette ; this is repeated two or three times so that the granules are thoroughly washed. This procedure is particularly necessary when there is mixed infection. Treatment of the granules with absolute alcohol for two minutes before transferring them to medium facilitates the isolation of the organism by destroying any associated pyogenic cocci (*vide* p. 573). Two agar plates are inoculated with the separated granules. One is incubated aerobically, the other anaerobically.

ACTINOBACILLUS (*Actinobacillus lignieresi*)

This organism has been cultivated from a large proportion of cases of bovine "actinomycosis." To such cases the term "actinobacillosis" has been frequently applied. In contrast to the actinomyces this organism does not usually invade bones and shows a marked tendency to spread by lymphatics. In such cases typical mycelial organisms cannot be detected in the tissues and the granules may be composed almost entirely of "club" formations. No mycelial organism can be cultivated, but on ordinary media under aerobic conditions a small Gram-negative bacillus develops. This organism is about 1·5μ in length and is non-motile. The designation *Actinobacillus* has therefore been applied to it. In shake cultures in glucose-agar elongated filamentous but unbranched forms are noted. The colonies are small, circular and translucent. Glucose, maltose, saccharose and certain other sugars are fermented. Inoculation of cultures into cattle and guinea-pigs reproduces lesions characteristic of actinomycosis with typical colonies or granules in the tissue.

Actinobacillosis has also been described in sheep, and strains of the actinobacillus isolated from these animals appear to be similar to those of bovine origin.

OTHER PATHOGENIC ACTINOMYCETES

Apart from the typical actinomycosis, granulomatous and suppurative conditions occur in animals and man, due to infection by mycelial organisms, which differ biologically from the actinomycetes described above. The following organisms may be taken as examples.

EPPINGER'S STREPTOTHRIX (*Actinomyces asteroides*)

Originally isolated from a brain abscess. The filaments are relatively broad (1μ in diameter) and very readily break up in culture into bacillary forms. They stain Gram-positively and are slightly acid-fast. This organism can be cultivated aerobically on ordinary medium as a friable, white, dry, wrinkled or nodular growth, which later becomes pigmented (yellow or pink).

ACTINOMYCES NOCARDII (*Actinomyces farcinicus*)

The organism of bovine " farcy," in which superficial lymph glands become swollen and ulcerate through the skin.

The organism shows mycelial formation, but in culture readily fragments into shorter bacillary and oval forms. It is Gram-positive and tends to be acid-fast. It grows aerobically at 37° C. on ordinary media, producing raised irregular greyish-white colonies after two to three weeks.

Guinea-pigs are susceptible to experimental inoculation and develop nodular or tubercle-like lesions. In cattle, subcutaneous injection leads to a localised abscess which breaks through the skin and produces a chronic ulcerated lesion. Rabbits are not susceptible to inoculation.

ACTINOMYCES (or ACTINOBACILLUS) ACTINOIDES

This organism has been isolated from a pneumonic condition in calves, and presents some similarities to *Actinobacillus lignieresi*. In the tissues it appears as a slender Gram-negative bacillus. Cultures have been obtained by placing a piece of infected lung tissue in the condensation fluid of a tube of coagulated serum and then sealing the tube, the growth occurring after several days as small white flocculi in the fluid. Microscopical examination of such cultures has revealed filamentous and club-shaped forms. The organism has also been cultivated on the surface of agar containing a piece of sterile guinea-pig spleen and with calf serum added, the colonies being small, coherent and slightly yellowish in colour.

THE ORGANISMS OF MYCETOMA OR MADURA FOOT

Mycetoma is an infective granuloma localised usually to the tissues of the foot, and exhibiting no metastases. The condition occurs only in certain tropical and subtropical countries (*e.g.* India, some parts of Africa, etc.).

In the tissue lesion and pus, granules or colonies are noted as in the case of actinomycosis. These granules vary in colour; in some cases they are white or yellow ("pale variety"), in others black ("melanoid variety").

The *pale granules* usually represent colonies of an actinomyces-like organism—*e.g. Actinomyces madurae*. Morphologically this organism resembles the classical actinomyces, but clubs are less frequently noted.

Cultural characters.—It is a strict aerobe. The optimum temperature is about 37° C.. On nutrient agar the growth consists of circular raised colonies like those of the actinomyces, at first yellowish, later pinkish.

Other species of actinomyces have also been reported in cases of mycetoma.

The *black granules* in mycetoma represent true fungi, which have been designated under the generic name of *Madurella*.

The colonies consist of a mycelium of branching, septate hyphae 3–8μ in breadth, and contain a considerable amount of black pigment.

This type of organism can be cultivated on nutrient agar, and old cultures show the black pigmentation.

A number of different varieties have been described and this group has been regarded as related to the genus *Aspergillus*.

LEPTOTHRIX

An organism of this type (*vide* p. 5) is a common inhabitant of the mouth cavity, and may be detected in films made from the secretion between the teeth or deposits of tartar. It is designated *Leptothrix buccalis*. Pathogenic properties have been claimed for it, but its invasive power is probably slight.

Leptothrix types have also been reported in suppurative lesions in the region of the mouth and throat.

BACILLUS RHUSIOPATHIAE (*Erysipelothrix rhusiopathiae*)

The causative organism of swine erysipelas.

Morphology.—Slender, Gram-positive, non-motile rod-shaped organism 1–2μ by 0·2–0·4μ, occurring singly and in chains. In culture media, longer and filamentous forms are observed. True branching has been described.

Culture.—Growth occurs on ordinary media even at room temperature, though the optimum is about 37° C.. The organism shows micro-aerophile characters and in stab cultures in agar or gelatin does not grow on the surface. Surface growths may be obtained under anaerobic conditions. In gelatin-stab culture a line of growth occurs along the needle track with lateral spikes or disks radiating from the central growth. Surface colonies on plates are of two types: one exceedingly minute and dewdrop-like, with a smooth surface; it does not exceed 0·5 mm. in diameter when growing on agar. The other is larger and has a granular appearance.

Experimental Inoculation.—Mice, rats, rabbits and pigeons are susceptible to inoculation. Mice and pigeons are specially susceptible, and usually die of an acute septicaemia within four or five days after experimental inoculation. Subcutaneous injection in rabbits produces a spreading inflammation and oedema with a fatal result. Experimental inoculation (with cultures) in swine reproduces the disease as it occurs naturally. The smooth-colony type of culture is the more pathogenic.

Occurrence.—The bacilli can be observed in the characteristic skin lesions, and in internal organs—*e.g.* lungs, spleen and kidney. In some cases there is a marked septicaemic condition and the organism is detectable in blood films, particularly in leucocytes. In the chronic form of the disease, in which a " verrucose endocarditis " occurs, the bacilli may be confined to the cardiac lesions.

Artificial *immunisation* against the disease has been carried out by the injection of immune serum immediately followed by injection of a virulent broth culture. Immune serum is also used for therapeutic purposes.

For diagnostic purposes an attempt should be made to cultivate the organism from lesions and in acute cases from the blood; inoculation tests should also be carried out in mice or pigeons. An agglutination test is applicable.

Cases of human infection (" erysipeloid ") by this organism have been recorded. This is usually contracted through abrasions of the skin when infected carcases are handled, *e.g.* by abattoir workers. The lesion is situated on the hand or forearm.

B. rhusiopathiae may occur in apparently healthy pigs, and has been isolated from the tonsils, intestines and faeces.

A similar organism, *Bacillus murisepticus* (*Erysipelothrix muriseptica*), is responsible for epizootic septicaemia in mice.

THE ORGANISM OF BOVINE PLEURO-PNEUMONIA

This organism was originally classified with the filterable viruses in view of its ability to pass the coarser filters ; it is, however, within the range of microscopic visibility and can be cultivated readily on artificial medium. The disease is transmissible experimentally in cattle by subcutaneous inoculation with exudate from the pulmonary lesions and also by the injection of cultures.

The morphology, which is best studied in impression preparations from cultures (p. 226), depends on the stage of growth, and diverse forms have been observed. Successive phases have been described : a granular phase in which the organism appears coccoid or cocco-bacillary, not exceeding $0{\cdot}4\mu$ in diameter ; a filamentous phase produced by the enlargement of the granules, their peripheral budding and the separation of the buds which remain attached to the parent structure by a delicate filament ; a mycelial phase developed by the formation of new filaments which produce a branching meshwork ; later the filaments seem to divide or their protoplasm becomes condensed into chains of coccus-like forms ; finally, the chains disintegrate into granular forms like those of the first phase. Ring forms, vibrionic forms and large oval bodies have also been observed. The filterability of the organism is probably due to the ability of the small granular bodies to pass through a coarse filter.

The organism is best stained by Giemsa's stain, heat being applied. It is Gram-negative.

Cultures can readily be obtained aerobically at 37° C. in serum-broth or on serum-agar (10 per cent. of horse or ox serum). Growth is visible in two to five days : in broth as a general cloudiness ; on agar as very minute droplet-like colonies which develop a raised centre ; these later become larger (1 mm. diameter), white, umbilicate and somewhat tenacious. Cultures remain viable for several weeks. Cultivation leads to attenuation of virulence. The organism is killed within one hour by heating at 58° C..

Cattle can be immunised against the disease by inoculating infective material or cultures into the point of the tail : injection in this situation produces a non-fatal infection which is followed by immunity for at least a year. The serum of immune animals contains protective antibodies.

Ledingham has suggested that the organism belongs to the *Actinomycetaceae.*

An organism similar to that of pleuro-pneumonia has been found as a supposed symbiont in cultures of *Streptobacillus moniliformis* (p. 502). The significance of such occurrence is obscure. A similar organism may also occur in a pulmonary infection of rats, arthritis in these animals, and in "rolling" disease of mice.

The filter-passing organisms isolated by Laidlaw and Elford from sewage (p. 598) are similar to the pleuro-pneumonia organism but show no antigenic relationship to it.

THE ORGANISM OF AGALACTIA

This infectious disease affects sheep and goats, and is transmitted by contagion. It is characterised by inflammatory lesions of the mammary glands, eyes and joints. The causative organism resembles the infective agent of bovine pleuropneumonia. It can be found in arthritic fluid. The organism will pass Berkefeld and the coarsest Chamberland filters. It can be cultivated on serum-agar. Recovery from the infection produces a lasting immunity.

BACILLUS FUSIFORMIS (*Fusobacterium plauti-vincenti*)

This organism is referred to (p. 545) as a concomitant of a spirochaete in Vincent's angina, and is found in various necrotic inflammatory conditions along with this spirochaete—*e.g.* ulcerative gingivitis and stomatitis, etc.—and occasionally in diphtheritic lesions of the throat.

Morphology.—It is a large, non-motile, fusiform bacillus, 5–14μ by 1μ.

Staining. — Gram - negative. The centre of the bacillus often stains less deeply than the poles, and a beaded or granular appearance may be noted.

Culture.—Anaerobe. It has been cultivated on a medium containing 1 part of blood to 3 of agar. The colonies are small white disks resembling a growth of streptococci.

BACILLUS NECROPHORUS (*Actinomyces necrophorus*)

This organism is responsible for diphtheritic and necrotic lesions ("necrobacillosis") in various animals—*e.g.* gangrenous dermatitis of equines, calf diphtheria, "foot rot" of sheep, necrotic stomatitis of pigs, liver abscesses in various domesticated animals.

The organism appears in the form of elongated slender filaments varying in length and attaining sometimes to 50 or even 100μ. Branching has occasionally been described. The filaments are Gram-negative and show a characteristic beaded appearance when stained by the ordinary stains. In addition to the filamentous form, the organism may be seen as small Gram-negative bacilli. Growth is obtained at an optimum temperature of 34°–36° C. on serum-agar under strictly anaerobic conditions. The colonies are small, white opaque disks with projecting wavy filaments. Cultures yield a characteristic "cheese-like" odour especially in a milk medium. Indole is formed. Gelatin is not liquefied.

Rabbits and mice are highly susceptible to inoculation. Subcutaneous injection in rabbits produces an initial focus of necrosis at the site of inoculation and the animal dies in one to two weeks.

For diagnostic purposes stained films made from the edges of the necrosed tissue are examined.

Direct cultivation is difficult owing to the large numbers of other organisms present in the lesions. Pure cultures can be obtained readily by inoculating rabbits or mice from the necrotic tissue and isolating the organism on serum-agar from the inoculated animal at autopsy.

This type of organism has occasionally been isolated from suppurative lesions in the human subject. It may occur in septic wounds associated with sloughing of the tissue. *B. necrophorus* has been found in the mouth, intestine and female genital tract of healthy persons. Strains of human origin appear to be only slightly pathogenic to animals. (The question of the relationship of this organism to *B. fragilis* and *B. fusiformis* is referred to on p. 470.)

CHAPTER XX

THE PATHOGENIC AND COMMENSAL SPIROCHAETES

TREPONEMA PALLIDUM

(Spirochaeta pallida)

The causative organism of syphilis.

Morphology.—An exceedingly delicate, spiral filament 6–14μ (av. 10μ) by 0·2μ, with six to twelve coils which are comparatively small, sharp and regular. The length of the coils is about 1μ and the depth 1–1·5μ. The ends are pointed and tapering. The organism is feebly refractile, and in the unstained condition requires dark-ground illumination for its demonstration (*vide* p. 69).

Treponema pallidum was originally described as having terminal flagella. Later it was supposed that the organism was devoid of flagella and the tapering ends were referred to as " terminal filaments." Recently in specially stained preparations definite terminal flagella have been demonstrated, and what appear to be lateral flagella have been revealed by the electron microscope.

In addition to the typical form, as described, some variation in morphology may be observed : the number of coils to the unit of length may be more or less than normal, the filament may be thicker than normal in whole or part and the coils may be shallower and less regular than usual.

The spirochaete shows rotatory corkscrew-like motility, and also movements of flexion. The coils remain relatively rigid, but there may be some expansion and contraction. Its progression is relatively slow as compared with many of the motile bacteria.

Multiplies by transverse binary fission.

Division into four and even smaller fragments has also been described. Some observers have claimed that a granule or granules may be split off, remaining attached by a delicate thread before final separation. This granular form has also been regarded as a phase in the life history of the organism.

Staining.—*Treponema pallidum* cannot be demonstrated by the ordinary staining methods. It can be stained by Giemsa's solution applied in a 1 in 10 dilution over a prolonged period (twenty-four hours) or in a 1 in 2 dilution for an hour, and appears faint pink in colour. For the demonstration of this organism in films, Fontana's silver impregnation method (*vide* p. 221) is one of the best available in routine work. In tissues, the spirochaetes can be stained by Levaditi's silver impregnation method (p. 222).

Culture.—According to the claims of Noguchi and others *Treponema pallidum* has been grown anaerobically in the Smith-Noguchi medium (*vide* p. 151), but most workers have completely failed to cultivate the organism. Noguchi's technique of isolating pure cultures was as follows. Deep tubes of 2 parts agar and 1 part ascitic fluid were prepared, with a piece of sterile tissue added (*e.g.* rabbit kidney). The medium was covered with a layer of sterile vaseline. The tubes were inoculated from exudate with a capillary pipette (stab inoculation). Both the spirochaetes and the bacteria present in the material grew along the line of inoculation, but later the spirochaetes spread out from the stab and formed a haze in the medium. The tube was cut, and transplants were made in Smith-Noguchi medium, without carrying over any of the other bacterial growth.

Viability apart from the body is feeble. This spirochaete is a strict parasite; it dies rapidly in water and is very sensitive also to drying. It is easily killed by the usual antiseptics and by heat (even at 41·5° C. in an hour).

Occurrence.—In the primary stage, spirochaetes are present in large numbers in the chancre and in the exudate from it, but as the sore tends to heal they become less numerous, and may not be demonstrable in the exudate. They are present also in the buboes.

In the secondary stage, spirochaetes have invaded the blood stream and become widely distributed in the body. They are present in the roseolar skin lesions, mucous patches and condylomata, and have been demonstrated even in the blood.

In the tertiary stage, they are less easily demonstrated in lesions, but can be observed by suitable staining methods in the periphery of gummata, in arterial lesions, etc..

In general paralysis of the insane, *Treponema pallidum* has been demonstrated in the cerebral cortex.

In congenital syphilis, spirochaetes are found in certain internal organs—*e.g.* liver—often in very large numbers. They are present also in the skin lesions, the blood, and the mucosa of the intestine and bladder.

Spirochaetes have also been demonstrated in the placenta.

Animal Inoculation.—Monkeys have been infected experimentally by inoculation of a scarified area on the eyebrows and genitals, or by implanting tissue from a syphilitic lesion under the epidermis. The anthropoid apes are the most susceptible, and lesions typical of primary and secondary syphilis may result in these animals. Rabbits can also be infected in some cases by inoculation in certain sites : inoculation into the anterior chamber of the eye produces keratitis and iritis ; intratesticular injection leads to a syphilitic orchitis ; and inoculation of the skin of the scrotum may set up a chancre-like sore. Metastatic lesions may succeed the primary infection.

Inoculation of mice produces no lesions and though infection takes place it is symptomless and apparently latent.

SEROLOGICAL REACTIONS

Wassermann Reaction.—Reference has been made to this manifestation of syphilis on pp. 263–278, and the technique has been described.

The reaction appears in about two to four weeks after the onset of the primary lesion, but its development may sometimes be delayed. In secondary syphilis the reaction is usually well marked and fairly constantly present. It is less frequently positive in the latent stage (25–50 per cent. of cases) and in tertiary cases (75 per cent.). In general paralysis and in many cases of locomotor ataxia, the reaction is positive both when tested with blood and with cerebro-spinal fluid. In cerebro-spinal syphilis the spinal fluid may react positively even when the blood yields a negative reaction. Active cases of congenital syphilis usually exhibit a strongly positive reaction.

As the result of antisyphilitic treatment the Wassermann reaction may become negative, but often temporarily, and the reaction reappears when treatment is stopped.

Positive reactions have been recorded in leprosy and malaria, but in assessing the significance of these results, it must be remembered that concomitant syphilis may be responsible for the Wassermann reaction noted in other diseases. It has been supposed that a positive reaction may develop in leprosy apart from associated syphilis, but there is no clear evidence that leprosy induces a positive Wassermann reaction. A positive Wassermann reaction occurs in yaws, and has been recorded in cases of relapsing fever and trypanosomiasis.

The Flocculation Reaction is dealt with on p. 278. It follows very closely the Wassermann reaction in its occurrence, and corresponds quantitatively with the latter. The two reactions, however, are not in all cases parallel in their occurrence and degree.

It has recently been shown that syphilitic serum agglutinates strongly the "Reiter" strain of cultivated spirochaete supposed to be originally *Tr. pallidum* (Gaehtgens reaction), and likewise yields a complement-fixation reaction with this strain. The suggestion has been made that the spirochaete is a more complete and more sensitive antigen for the syphilis serum reactions than the usual lipoid suspension. The practical application of these observations requires further confirmation.

DIAGNOSIS

In the primary stage, when there is an ulcerated sore, *Tr. pallidum* can usually be demonstrated in the serous exudate from the lesion. The dark-ground illumination method is the most suitable technique for the purpose, and provides a convenient means of rapid diagnosis. Failing this, Fontana's staining method can be used.

Tr. pallidum is recognised by its special morphological features, and must be carefully differentiated from other spirochaetes found in ulcerating sores—e.g. *Tr. gracile*, etc. (*vide infra*).

Directions for obtaining a specimen of exudate from a syphilitic sore for microscopic examination : The serous exudate should be obtained from the tissue, and should not include surface organisms, as other spirochaetes which may be confused with the *Tr. pallidum* are frequently present. The presence of excessive numbers of red blood corpuscles in the specimen is also to be avoided, as they tend to obscure the spirochaetes. If a local antiseptic has been used it may not be possible to find spirochaetes until a wet dressing of gauze soaked in sterile saline solution has been applied to the sore for twenty-four to forty-eight hours. It is to be noted also that antisyphilitic treatment, initiated before the examination, diminishes the likelihood of successful microscopic diagnosis. The sore is cleansed with a swab soaked in warm saline solution, and the margin is then scraped lightly with some blunt instrument to abrade the superficial epithelium. On squeezing the base of the chancre, serum exudes, and if blood-stained, should be removed with dry gauze until clear exudate can be obtained. Some of this is then collected in one or two capillary tubes. Both ends of the tube are sealed in a flame, and the specimens are submitted for examination. Another method of obtaining exudate is to apply spirit to the sore for a minute, and allow the surface to dry ; this leads to an exudation of serous fluid, which is collected and examined.

When the primary sore is in process of healing, microscopic examination of the exudate may yield negative results. At this stage spirochaetes may be found in the fluid aspirated from the buboes by means of a syringe.

In the secondary stage spirochaetes may also be demonstrated in the serum from the skin eruption, and in the exudate from mucous patches, etc.. Serum can be obtained from the skin eruption by scarifying and " cupping " with a test-tube.

After about two weeks from the onset of the primary sore, the Wassermann reaction can be employed for diagnosis. The reaction becomes progressively more pronounced with the advance of the disease, and is markedly positive in the secondary stage.

Owing to the fact that the reaction may be slow in developing, if at first a negative result is elicited in the primary stage, it is essential to repeat the test before excluding syphilis. A negative reaction in a suspected case of secondary syphilis is highly significant in excluding syphilitic infection, but in supposed latent or tertiary cases a negative result does not exclude the disease.

In cerebro-spinal syphilis both the blood and spinal fluid should be tested.

In dealing with cases of congenital syphilis in young infants, the mother's blood should be tested if there is any difficulty in obtaining a specimen of blood from the child.

It may be said that the Wassermann reaction, carried out by a reliable technique, is generally diagnostic of syphilis among the diseases of a temperate climate.

The Flocculation reaction may for routine purposes be substituted for the Wassermann test, and is simpler to carry out.

TREPONEMA PERTENUE

The causative organism of Framboesia or Yaws, a tropical disease pathologically and clinically resembling syphilis, though differing in its highly contagious character.

Morphology.—Practically identical with the *Tr. pallidum.* When first described it was regarded as more slender than *Tr. pallidum*—hence the designation.

Its occurrence in lesions corresponds to that of the *Tr. pallidum,* and the diagnosis of the infection is carried out as in syphilis, by demonstrating the spirochaete in the papules or ulcers. The Wassermann reaction is positive.

The infection is experimentally transmissible to monkeys and rabbits as in the case of syphilis. Certain differences have been noted in the experimental lesions as compared with those produced by *Tr. pallidum.*

OTHER TREPONEMATA

Treponema calligyrum (or *gracile*).—This organism may occur in the secretions of the genitals, and morphologically resembles *Tr. pallidum.* Its differentiation from the latter is therefore of practical importance in syphilis diagnosis. It is not usually found if care has been taken to obtain serum from below the surface of the chancre (*vide supra*). It is thicker than *Tr. pallidum* and its spirals are narrower; by the dark-ground illumination method it appears " glistening," whereas *Tr. pallidum* is " dead white "; it stains more readily than *Tr. pallidum* by Giemsa's method.

Treponema microdentium.—This organism flourishes in carious teeth, and may be found in the secretion between the teeth. It closely resembles *Tr. pallidum* in morphology, but is shorter (3–10μ), and the coils are shallower. It is more easily stained by the ordinary methods than *Tr. pallidum.*

Treponema mucosum.—Similar to *Tr. microdentium* in morphology, but is stated to have the property of producing mucin.

Treponema macrodentium.—Occurs in the mouth like *Tr. microdentium.* It also resembles *Tr. pallidum*, but is larger and thicker, with larger and less regular coils. It is more easily stained than *Tr. pallidum* and is coloured blue by Giemsa's method.

Treponema cuniculi.—Associated with an infectious disease of rabbits, which usually takes the form of a chronic local and superficial infection of the genitals. The spirochaetes can be demonstrated in the exudate from the lesions and in tissue sections. They are morphologically identical with *Tr. pallidum.*

SPIROCHAETES OF THE "REFRINGENS" TYPE

Borrelia (or *Spironema*) *refringens*

These are large, motile, refractile spirochaetes (about $10–30\mu \times 0{\cdot}5–0{\cdot}75\mu$) with irregular wide and open coils which are relatively few in number. They are easily stained by the ordinary methods and are Gram-negative. They occur as commensals on various mucous membranes—*e.g.* mouth—and in gangrenous and ulcerative conditions on the surface of the body, the mouth and throat, and the genitals. A spirochaete of this form occurring in the mouth has frequently been designated by the specific name *buccalis.* This type of organism may also be found in the surface exudate of a syphilitic sore, and has to be differentiated morphologically from the *Tr. pallidum.*

"*Borr.* (or *Sp.*) *vincenti*" occurs in a pseudo-membranous condition of the throat — Vincent's angina. It resembles *Borr. refringens*, but is sometimes smaller ($5–10\mu$) and more delicate. It is generally associated with a large fusiform bacillus—*B. fusiformis* (*vide* p. 536). Films from the throat secretion, stained by dilute carbol fuchsin or methyl-violet, show large numbers of spirochaetes.

A similar spirochaetal infection associated with *B. fusiformis* occurs in balanitis gangrenosa, ulcerative stomatitis and gingivitis and a chronic ulceration of the skin of tropical countries (ulcus tropicum).

A similar infection has also been observed in putrid bronchitis and empyema, gangrene of the lung and pulmonary abscess.

Such infection by *Borr. vincenti* and *B. fusiformis* is generally associated with necrosis of tissue, pseudo-membrane-formation, and a putrefactive odour.

THE SPIROCHAETES OF RELAPSING FEVER

BORRELIA (or SPIRONEMA) OBERMEIERI (or RECURRENTIS)

The causative organism of European relapsing fever.

Morphology and Staining.—This organism is a spiral filament, varying in length, as a rule, from 10 to 20μ, and about 0·3μ broad, with about five to seven fairly regular coils 2–3μ long by 1μ in amplitude. Active motility of a rotatory or oscillating type is noted in fresh preparations. Multiplication is by transverse fission.

This spirochaete stains readily with a Romanowsky stain (*e.g.* Leishman's), and may exhibit uniform staining or beading. It can be stained also with carbol fuchsin, and is Gram-negative. In fresh preparations of blood it can be seen with the ordinary microscope, but dark-ground illumination is more suitable for its demonstration in the living state. Silver impregnation methods may also be used for demonstrating the spirochaete in films or tissues (*vide* p. 221).

Culture.—Artificial cultures were first obtained anaerobically in Smith-Noguchi medium, citrated blood containing spirochaetes from an infected animal —*e.g.* a white rat—being used as the inoculum.

More recently cultures have been obtained in other media but the organism does not readily adapt itself to artificial growth in the laboratory: (1) horse serum diluted with 2 parts of saline solution, containing 1 per cent. peptone broth[1] and (for subcultures) a drop of rabbit blood, the medium being covered with a paraffin seal; (2) 20 per cent. rabbit serum with 80 per cent. Hartley's broth in tubes to each of which 1 gram of coagulated egg albumin is added; a vaseline seal is superimposed and the cultures are incubated at 30° C.; (3) some egg albumin is placed in a test-tube and coagulated by heat in the form of a slope (*vide* p. 101); 5 c.c. of horse serum diluted 1:10 or rabbit serum diluted 1:5 are then added, the serum having previously been heated at 58°–60° C. for one hour; the medium is covered with a layer of sterile vaseline; before an inoculation is made a drop of fresh rabbit or human blood is added.

Occurrence.—This organism is present in the peripheral blood during the pyrexial stage of the illness, and can be detected in blood films. When defervescence occurs it disappears from the blood, but may still be present in considerable numbers in the spleen, where it is phagocytosed by large mononuclear cells.

It is transmitted from person to person by the body louse, *Pediculus humanus corporis.* After this insect has sucked blood from the infected individual the organisms are demonstrable in the stomach for a day, and then disappear. They reappear after about six days in the body cavity and become widespread throughout the body of the insect. Infection results either through the contamination of the bite wound with the infective excreta of the louse, or by the crushing of the infective lice with the fingers in the act of scratching and by the simultaneous inoculation of the abrasions.

Animal Inoculation.—Monkeys, white mice and white rats can be infected experimentally by subcutaneous injection of blood from a case of relapsing fever. The guinea-pig is not susceptible.

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[1] 1 c.c. of broth containing 10 per cent. peptone to 10 c.c. of diluted serum.

BORRELIA (or SPIRONEMA) DUTTONI

The organism of West African relapsing fever (African Tick Fever).

This organism is morphologically and biologically similar to *Borr. obermeieri*, but probably represents a separate species. Its distribution in the disease is also similar, but it is transmitted by a tick (*Ornithodorus moubata* and other species). Infection probably results from the contamination of the bite-wound by the infective excreta of the tick. It has been suggested that in the tick the organism goes through some stage in a life-cycle. "Chromatin" granules have also been observed in the spirochaete; these are extruded, and have been regarded as a phase in the life history of the organism. These granules have been noted in the Malpighian tubules of infective ticks. Infectivity may be transmitted to a second generation from the female tick.

Borr. duttoni is pathogenic to monkeys and certain laboratory animals (*e.g.* rat, mouse). It possesses a greater virulence for monkeys and other animals than *Borr. obermeieri.*

Other relapsing fever spirochaetes.—The spirochaete of North American relapsing fever resembles *Borr. obermeieri*, but has been regarded as a separate species on the basis of immunity reactions. It is designated *Borrelia* (or *Spironema*) *novyi*. It is louse-borne.

The organism of Indian relapsing fever also corresponds in its biology and pathogenesis to *Borr. obermeieri*. It has been named *Borrelia* (or *Spironema*) *carteri*, but it is doubtful if the Indian strains can be differentiated from the European. This infection is also louse-borne.

Louse-borne spirochaetal relapsing fever similar to the Indian form occurs in various parts of Asia, but in Central Asia tick-borne relapsing fever is also present.

The common form of relapsing fever in North Africa is louse-borne. In tropical Africa the prevalent type is tick-borne (African Tick Fever, *vide supra*), though louse-borne infections occur in West Africa.

In Central and South America both louse-borne and tick-borne forms of the disease have been observed.

Immunity to the Relapsing Fever Spirochaetes.—Recovery from an attack is associated with the appearance of agglutinating and lytic antibodies in the blood serum, and in this way the general infection is temporarily checked though spirochaetes may still persist in the internal organs. It would appear that the relapse is due to antigenic variation in the surviving spirochaetes. The variant strain uninfluenced by the antibodies produced towards the parent organisms is able to flourish and reinfect the blood. Multiple relapses, as in African relapsing fever, are apparently due to repeated antigenic variation. In Indian relapsing fever in which there are usually two attacks only, the relapse-strain transmitted experimentally to animals reverts to the serological characters of the original strain after producing a first attack in the animal.

DIAGNOSIS OF RELAPSING FEVER

During the pyrexial phases, the spirochaetes can frequently be demonstrated in the blood, but not during apyrexial intervals.

Thin or thick blood films are made as in malaria diagnosis, and stained by Leishman's method (*vide* pp. 217, 579, 580).

Some workers prefer to stain the films with dilute carbol fuchsin.

If a drop of blood is mounted on a slide under a cover-slip and examined with the oil-immersion lens, the spirochaetes may be detected in the unstained condition and show active movement. A more satisfactory method of demonstrating them, however, is by dark-ground illumination.

If spirochaetes are not detectable, inoculation of white mice with blood drawn from a vein may reveal the infection, the organisms appearing in considerable numbers in the blood of the animals.

BORRELIA (or SPIRONEMA) THEILERI

This spirochaete is responsible for a blood infection occurring in cattle, sheep and horses in Africa. The disease is of a comparatively mild type. The organism appears as a spiral filament 10–30μ by 0·25–0·3μ and is actively motile when seen in fresh preparations of blood. It is transmitted by a tick (*Margaropus decoloratus*).

BORRELIA GALLINARUM (or SPIRONEMA ANSERINUM)

This organism produces a general blood infection in geese and fowls. It can be seen in the blood of the infected bird both in unstained preparations and in films stained by a Romanowsky stain or dilute carbol fuchsin. It is a motile spiral organism 10–20μ in length by 0·3μ in breadth, and exhibits several coils. Artificial cultures have been obtained in Smith-Noguchi medium. The disease is transmitted by ticks—e.g. *Argas persicus*—and a granular phase has been described analogous to that observed in the case of *Borr. duttoni* (*vide supra*). By experimental inoculation various species of birds may be infected, but mammals are not susceptible.

LEPTOSPIRA ICTEROHAEMORRHAGIAE

The causative organism of Infectious Jaundice (Weil's Disease).

Morphology and Staining.—A leptospira (*vide* p. 14) about 7–14μ long by 0·15μ broad. The coils are very numerous and so fine that they are difficult to demonstrate in stained preparations, though quite obvious by dark-ground illumination. In addition to these " elementary " spirals, larger " secondary " coils may be seen, especially in stained films. Hooked ends are a characteristic morphological feature. In culture the organism tends to be longer than in the tissues. Active movement is observed in fresh preparations examined with the dark-ground microscope. The movement is both rotatory and undulating.

The organisms can be stained by Giemsa's solution (as in the case of *Tr. pallidum*) but the silver impregnation methods of Levaditi and Fontana give the best results.

Culture.—*L. icterohaemorrhagiae* can be cultivated readily in Noguchi's leptospira media (*vide* p. 152), and grows just below the surface, *i.e.* like a microaerophile organism. Schüffner's or Fletcher's medium also serves very well for the cultivation of this spirochaete (*vide* pp. 152, 153). The optimum temperature is from 25° to 30° C., but growth may also occur at 37° C..

Occurrence.—The organisms are present in the blood during the first six days of the illness, and though scanty, have occasionally been demonstrated microscopically in blood films. Later they disappear from the blood. They are present in the liver often in considerable numbers, and, particularly during the later stages of the disease, in the kidneys, when they can be detected in the urinary sediment.

Leptospira icterohaemorrhagiae occurs in wild rats and field mice, which act as reservoirs or carriers of the infection. In these animals the spirochaetes are present in the kidneys, and are excreted in the urine. In this way soil, water, food, etc., are contaminated. Infection of the human subject may occur by the alimentary tract, but it has been shown that the organisms can pass through the skin, possibly more readily when there are fissures or abrasions or when it has become sodden by continuous wetting. In Japan, epidemics of infectious jaundice have been specially noted among workers in wet mines. Similarly, during the war of 1914–18 outbreaks occurred among troops in wet trenches. The infection may follow bathing in stagnant bathing-pools contaminated by rats, and accidental immersion in canal water has also been the cause of the disease. In this country the disease has affected miners working in wet mines, sewer workers, fish curers, etc.. Transmission from the urine of rats harbouring the leptospira is undoubtedly an essential factor in the spread of the disease, and invasion occurs through skin abrasions or the intact skin.

Saprophytic leptospirae similar in morphology to *L. icterohaemorrhagiae* have been observed in water (e.g. *L. biflexa*), and the question has arisen whether these organisms are potentially pathogenic. Infectious jaundice has been produced in experimental animals by inoculation with apparently saprophytic leptospirae, but generally such organisms have proved non-pathogenic.

L. icterohaemorrhagiae infects dogs, producing the condition known as "Yellows" which is pathologically similar to human infectious jaundice.

The infection has also been observed in the fox.

DIAGNOSIS

During the first six days of the disease 5 c.c. of blood are withdrawn by vein puncture, citrated (*vide* p. 320) and injected intraperitoneally into a guinea-pig. In typical cases the inoculation produces death of the animal in eight to twelve days with a marked jaundice and with haemorrhages in the lungs, under the serous membranes and in the muscles. The leptospirae are present in large numbers in the liver and kidneys, and can also be found in various other organs and in the blood.

The methods of demonstration in the stained and unstained conditions have been referred to above. In early cases the organism may also be cultivated directly from the blood.

If a case is met with only at a later stage—*e.g.* after ten days from the onset—the urine is centrifuged and the sediment examined by the dark-ground microscope. Guinea-pigs should also be inoculated intraperitoneally with the centrifuged deposit.

The organism is present in the urine by the twentieth day in nearly every case; it may persist till the fortieth.

In the urine the organism often presents an atypical appearance and cannot be identified readily as a leptospira; this has been attributed to the bile or acid in the urine. Inoculation of animals with urinary sediment may not produce the characteristic disease; jaundice may be absent though lung haemorrhages are a more constant feature. In such animals the leptospirae may be relatively scanty in the tissues.

It may be noted here that the elementary coils of the dying organism may become unwound or opened out, so that it presents an appearance more like that of a treponema.

Serological Diagnosis.—Leptospiral infection not infrequently occurs without the typical signs and symptoms, and a diagnosis can only be made by laboratory methods. In such cases serum reactions have proved most valuable for diagnostic purposes. Antibodies, including agglutinins, appear in the serum of infected persons after about seven days from the onset of the disease, and progressively increase in amount.

Microscopic agglutination test.—Young living cultures in a fluid serum medium (*e.g.* Schüffner's or Fletcher's, *vide* p. 153) grown for four days at 32° C. and then for three days at room temperature may be used for the test. Alternatively a formolised culture has been used but only pure formalin (analytical reagent) should be added, and its concentration must not exceed 0·5 per cent. Otherwise formic acid is liberated in the preparation and causes acid-agglutination. The formolised culture can be stored, and it is stated that if kept in bottles covered with black paper and in the dark remains satisfactory for several months.

A series of doubling dilutions of serum is prepared—*e.g.* from 1 in 5 to 1 in 640 or higher (*vide* p. 251); an equal volume of the culture is added to each and the mixtures are incubated for two hours at 32° C.. A loopful is then taken from each and placed on a slide, and the drops are examined (for agglutination of the spirochaetes) by dark-ground illumination with a dry dark-ground condenser and first a $\frac{2}{3}$ in. and then a $\frac{1}{6}$ in. objective. When young living cultures are used for the test, and if the serum reacts positively, agglutination is observed in the lower dilutions and lysis in the higher; with formolised cultures lysis is absent.

The titre of the reaction may rise to 1 in 1000 or over. A control must be included to ensure that any agglutination observed is not occurring independently of serum.

As there may be serological differences among strains of the leptospira it has been recommended by some workers that a polyvalent agglutinable antigen (prepared from multiple strains) should be used.

Macroscopic agglutination test.—A 4–7 days' culture in Fletcher's medium incubated at 30°–37° C. is used as the spirochaetal suspension. This culture may contain a certain amount of sedimented material and therefore the supernatant fluid should be pipetted off, taking care not to disturb the sediment on removal of the culture bottle from the incubator. To obtain satisfactory results the culture should be of such density as to show by dark-ground illumination with a $\frac{1}{6}$ in. lens not less than 40 leptospirae per microscopic field. The general technique of the test is similar to that of other agglutination tests (p. 251). Doubling dilutions of the serum are prepared ranging from 1 in 2 to 1 in 64, and to 0·2 c.c. of each of these is added 0·6 c.c. of the culture, the final dilutions ranging from 1 in 8 to 1 in 256. The mixtures are incubated for 2 to 4 hours at 37° C. when readings are made, and the tubes are also allowed to stand overnight for later readings. Visible agglutination in a 1 in 32 dilution may be regarded as significant ; but frequently the reaction occurs in much higher titres. A rising titre on repeated testing provides conclusive evidence of infection.

Adhesion phenomenon (*Rieckenberg Reaction*, thrombocytobarin reaction).—This reaction has also been applied in the serological differentiation of races of spirochaetes and by such means *L. icterohaemorrhagiae* can be distinguished from *L. biflexa* and *L. hebdomadis* (*vide infra*). When spirochaetes are acted on by a specific antiserum they undergo such change that particles present (*e.g.* blood platelets, bacteria, etc.) tend to adhere to them, the adhesion being easily observable microscopically by dark-ground illumination. The diagnostic test is carried out by mixing patient's serum with a young culture of the spirochaete, a suspension of a young culture of *B. coli*, and a 1 in 5 dilution of fresh guinea-pig serum (*e.g.* 0·2 c.c. of each) ; the mixture is incubated at 37° C. for thirty minutes and a microscopic preparation is then examined by dark-ground illumination. The adhesion of the bacteria to the spirochaetes constitutes a positive reaction. A control test with a known normal serum is also carried out.

OTHER PATHOGENIC LEPTOSPIRAE

Various leptospiral infections other than the typical infectious jaundice due to *L. icterohaemorrhagiae* have now been described and defined, and a group of leptospirae can be recognised, the individual members of which differ in pathogenicity to man and animals, serological characters, their natural hosts, etc.. The following exemplify the various types or species which have been identified in addition to *L. icterohaemorrhagiae.*

Leptospira hebdomadis.—An organism described as the cause of "seven-day" fever of the East. It is morphologically identical with *L. icterohaemorrhagiae.* In some cases it has been demonstrated in the peripheral blood, and at a later stage of the illness appears in the urine. It is pathogenic for *young* guinea-pigs, producing a febrile illness rarely associated with jaundice, and with less tendency to haemorrhages than other leptospiral infections. These animals may also recover from the infection. It is not virulent to rats or mice. This organism differs serologically from the other pathogenic leptospirae. It is harboured by a field mouse (*Microtus montebelloi*) and excreted in the urine as in the case of rats infected by *L. icterohaemorrhagiae.* The mode of infection is probably similar to that in infectious jaundice.

Leptospira autumnalis.—This organism has been found associated with a disease in Japan called Akiyami, or harvest sickness, and clinically resembling a mild infectious jaundice. Cases may be confused with "seven-day" fever, but *L. autumnalis* can be distinguished from *L. hebdomadis* by its high infectivity to guinea-pigs in which it produces typical haemorrhagic jaundice. It can be differentiated from *L. icterohaemorrhagiae* by serological reactions. The field mouse (*Microtus montebelloi*) is a reservoir of this infection. The organism has also been found in *Apodemus speciosus.*

Leptospira grippo-typhosa.—Has been described in "swamp fever" of Eastern Europe. It can be differentiated serologically from *L. icterohaemorrhagiae* and is only weakly pathogenic to guinea-pigs, though infective to mice.

Leptospira canicola.—This organism which is serologically distinct from *L. icterohaemorrhagiae* has been described in infectious jaundice of dogs, and has also been found in rare cases, mostly without jaundice, in the human subject. It appears to be of lesser pathogenicity to guinea-pigs than *L. icterohaemorrhagiae.*

Leptospira pyrogenes.—Has been reported in a febrile disease occurring in Sumatra associated with some degree of jaundice and albuminuria. This organism is pathogenic to guinea-pigs, but its effects are irregular. It has been isolated by cultivation from the blood. *L. pyrogenes* has been found to be serologically distinct from other races of leptospira.

Leptospirae described in Phlebotomus Fever (Sand-Fly Fever), *Dengue and similar diseases.*—From cases of Phlebotomus fever a leptospiral organism has been isolated by blood-cultivation in Noguchi's leptospira medium (Whittingham). The organism proved non-pathogenic to guinea-pigs. It has long been recognised that a sand-fly (*Phlebotomus pappatasii*) is the vector of the specific agent, and the experimental work on the disease points to its causation by a filterable virus.

A leptospira has also been described in Dengue. The organism was demonstrated in the blood at the commencement of the illness, and was found to be pathogenic to rabbits, producing pyrexia and blood infection. It has been stated that the injection of rabbits with crushed infective sand-flies produces a similar disease, and that the inoculated animals show the spirochaetes in their blood. Dengue is now generally regarded as a filterable virus disease (p. 632).

It seems probable that in the Mediterranean area and Near East leptospiral infections occur which simulate Phlebotomus fever and Dengue. These may be *L. icterohaemorrhagiae* infections of a mild type with absence of the typical jaundice, or may be due to different races of *Leptospira* of lower virulence for man and laboratory animals than *L. icterohaemorrhagiae*.

SPIROCHAETA MORSUS MURIS (SPIRILLUM MINUS)

The causative organism of rat-bite fever. Though originally described as a spirochaete, this organism conforms in its biological characters to those of a spirillum, and the alternative name *Spirillum minus* has been used.

It is a short spiral organism about 2–5μ in length and relatively broad with regular short coils numbering one for each micron of the length of the organism. Longer forms up to 10μ may also be observed. This organism is very actively motile, showing darting movements like those of a vibrio. Movement is due to terminal flagella, which are variable in number—from one to seven at each pole. In moving, the organism itself remains rigid and shows no undulation. It can be demonstrated easily by dark-ground illumination in fresh preparations in which its active movement is seen and its flagella are also observed. It is most readily stained by a Romanowsky stain (*e.g.* Leishman's), but can also be stained by the ordinary aniline dyes.

Successful cultivation has been claimed by certain workers using Smith-Noguchi or Shmamine medium. The latter is prepared by adding 0·5 gm. sodium nucleate to 160 c.c. of sterile horse serum ; carbon dioxide is passed through the mixture for a few minutes until the medium becomes transparent; it is then heated on three successive days at 60° C., and on the fourth day at 65° C. for ten minutes, when it becomes semi-coagulated.

In rat-bite fever the spirillum may be demonstrated in the local lesion, the regional lymph glands, and even in the blood, either by direct microscopic methods (*vide supra*) or by animal inoculation. Guinea-pigs, white rats and mice are susceptible to infection: the spirilla appear in the peripheral blood and can be detected easily by dark-ground illumination. Guinea-pigs develop a progressive disease and die of the infection; in mice the organisms gradually disappear from the blood without producing, as a rule, a lethal effect. If the spirillum cannot be detected microscopically in the local lesion, or if the original bite wound has healed, an enlarged lymphatic gland may be punctured by means of a hypodermic syringe; "gland juice" is aspirated and investigated by direct methods or animal inoculation.

Spirillum minus occurs naturally in wild rats and certain other wild rodents, producing a blood infection. Conditions similar to rat-bite fever have also been reported following the bites of cats and ferrets.

It should be noted that at least two different specific infections may result from rat-bite and may be designated clinically "rat-bite fever": the condition due to *Spirillum minus* (described above) and that produced by the so-called *Streptobacillus moniliformis* (p. 502). The former presents a highly characteristic clinical syndrome: a relapsing febrile illness with a local inflammatory lesion, enlargement of regional lymph glands and a macular skin eruption, all these lesions fluctuating in parallel with the temperature. *Spirillum minus* has been demonstrated by direct examination in the local lesion and glands, and even in the blood of cases. The infection is very amenable to treatment with organic arsenicals. Infection by *Streptobacillus moniliformis* is likewise an acute or subacute febrile condition and may be associated with a skin eruption (*e.g.* erythema multiforme), but involvement of joints, even resembling the polyarthritis of acute rheumatism, is a feature of this illness. It seems likely that the two conditions have been confused with one another in the past.

CHAPTER XXI

RICKETTSIA GROUP; BARTONELLA (OROYA FEVER)

THE generic name *Rickettsia* is applied to a group of organisms whose biological relationships are still undetermined, though they may be classified provisionally with the bacteria. These organisms flourish in the alimentary tract of certain blood-sucking arthropods (*e.g.* lice, fleas, mites, ticks and bugs) and several species have now been described. Some have no relationship to human or animal disease, but certain are pathogenic to the mammalian host of the ecto-parasite in which they are found and which thus acts as the vector of the infection. Thus, *Rickettsia prowazeki* constitutes the specific agent of typhus fever and is transmitted by lice which have fed on typhus cases.

While investigating the cause of typhus fever, Ricketts and Wilder found in the alimentary tract of infected lice small diplococcal or rod-shaped structures, about 1μ or less in their long diameter, which stained reddish or purple by Giemsa's method. These small bodies, now termed *Rickettsia* (or "Rickettsia bodies"), have been found in infected arthropods transmitting other diseases such as Trench fever and Rocky Mountain spotted fever, and are regarded as the aetiological agents of these diseases. Although rickettsia bodies are found constantly and in large numbers in infective arthropods, and infectivity coincides with their presence, similar bodies have been found in non-infective ectoparasites, but in relatively small numbers and very infrequently. (It was for this reason that Ricketts and Wilder hesitated to regard the *Rickettsia* as the cause of typhus fever.) These rickettsiae, e.g. *Rickettsia da rocha-limae* of lice, represent non-pathogenic species. In addition similar bodies have been found almost constantly in the intestinal tract of the bed bug (*Cimex lectularius*) and sheep ked (*Melophagus ovinus*). There is no doubt that rickettsia bodies are definite living structures capable of multiplication, and that they may possess infective powers. The pathogenic rickettsiae, though they may be grown in tissue cultures like the filterable viruses (p. 605), have not yet been cultivated on artificial media.

TYPHUS FEVER

(*Classical louse-borne typhus of Europe and Asia*)

This disease is transmitted by the louse *Pediculus humanus* (*corporis* and *capitis* varieties), and the infective agent is now generally accepted to be *R. prowazeki* (*vide supra*). The disease can be reproduced in monkeys by inoculation with blood from human cases. The infection can also be communicated to guinea-pigs by the intraperitoneal injection of patient's blood taken during the height of the illness. After an incubation period of about nine or ten days, the animal's temperature rises to 105°–106° F., and remains above the normal for four to eight days. The guinea-pig does not succumb to infection, and on recovery does not react to a second inoculation. If the animal is killed on the first or second day of the fever, the blood is infective for other guinea-pigs. At autopsy, the spleen is found to be enlarged and the peritoneum congested. The skin on histological examination shows the presence of an exanthematous reaction.

The infective agent is demonstrable (by experimental inoculation) in the blood, spleen, brain and other organs. It is easily destroyed by heat and antiseptics, but is resistant to drying. It is not filterable.

Brill's disease of New York is a mild form of typhus fever.

Rickettsia prowazeki.—Small diplococcal or rod-shaped bodies, usually 0·3–1μ, but are pleomorphic and may attain a length of 4–20μ. They are non-motile and stain reddish purple with Giemsa's stain. They stain very feebly with ordinary stains and are Gram-negative. Up to the present they have not been cultivated on artificial media, but multiply in endothelial cells of a tissue culture and in the chorio-allantoic membrane of the embryo chick (*vide* p. 606). Good growth has been obtained in the yolk sac of the fertile egg, suspensions from which being many hundred times more infective than other cultures. The rickettsia bodies are found in the alimentary tract of lice infected with the blood of typhus fever patients and are regarded as the cause of the disease. They may be found in large numbers in smear preparations (stained by Giemsa's or Castaneda's method—pp. 218, 226) from the gut contents of the lice seven to ten days after infection. They invade the lining epithelial cells, which become distended with them and finally rupture. They are present in the excreta of the louse and infect the human subject through the bite-wounds or through scratches and other abrasions.

The experimental disease referred to above has been produced by inoculation with the gut contents of infective lice. Rickettsiae are demonstrable microscopically in the vascular endothelium of the skin, brain, etc., in the human disease and in the experimental infection. When inoculated into the anterior chamber of the eye of the rabbit or guinea-pig the organisms produce an acute inflammatory condition and are found in large numbers in the cells of Descemet's membrane.

The serum of typhus patients contains O agglutinins for types of *B. proteus* designated "X2" and "X19," originally isolated from the urine of cases but the reaction is much stronger with the latter organism. This serological feature of the disease is a fairly constant one, and the agglutination reaction with X19 (Weil-Felix reaction) has therefore been utilised for diagnosis. The reaction is not present in conditions other than typhus fever and closely related infections (*vide infra*). The immunological significance of the phenomenon has not yet been elucidated (although it has been suggested that a specific polysaccharide substance is common to *B. proteus* X and *R. prowazeki*), but it is not to be interpreted as indicating any aetiological relationship of *B. proteus* to the disease.

DIAGNOSIS

The Weil-Felix reaction is performed in exactly the same manner as the Widal test (p. 250), except that a suspension of *B. proteus* X19 is used instead of the enteric organisms.

As the agglutinins are of the O type, an O culture should be used for the test (*B. proteus* O–X19). As this may revert to the H form it should be grown on dry agar and, if necessary, subcultures are made from non-spreading separate colonies (Felix).

The minimum titre acceptable for diagnostic purposes is 1 in 100, but agglutination frequently occurs in much higher dilutions. The reaction appears about the sixth or seventh day of the disease.

Reference has been made above to the recognition of *R. prowazeki* in lice and in tissues, and to the results of animal inoculation.

Rickettsial agglutination test—*vide* p. 564.

" MURINE " TYPE OF TYPHUS FEVER

This form of typhus was first recognised in the United States and in Mexico where it is designated " Tabardillo." The causative organism is designated *Rickettsia mooseri*. When infective material is inoculated into the peritoneum of the male guinea-pig an inflammatory reaction develops in the scrotal sac (Neill-Mooser reaction) with rickettsiae flourishing in the endothelial cells. This is not observed when inoculations are made from European typhus. The infection can be transmitted to rats in which the resistance has been lowered by X-radiation. Wild rats constitute a reservoir of the infection, and the rat flea (*Xenopsylla cheopis*) serves as the vector among rats, and from rats to man. Once established in the human subject the infection is transmitted by lice. The serum of cases (as in European typhus) agglutinates *B. proteus* X19. This form of typhus fever occurs also in Europe (*e.g.* Fièvre nautique of Toulon), Middle East and Malaya ("Urban typhus"), and other parts of the world.

It may be noted that in South Africa three rickettsial infections have been reported: louse-borne typhus, murine typhus and a tick-borne typhus-like condition (*vide infra*).

Rickettsial agglutination test for differentiation of classical and murine typhus—*vide* p. 564.

IMMUNITY

Recovery from typhus is followed by a lasting immunity and sera of convalescent persons have been used prophylactically in the incubation period. Some success has been achieved in prophylactic immunisation with killed suspensions of rickettsiae obtained by emulsifying in phenol-saline the intestines of artificially infected lice, *e.g.* Weigl's vaccine. A vaccine has also been produced from suspensions of rickettsiae grown in the yolk sac of the fertile egg (Cox's vaccine).

OTHER INFECTIONS DUE TO RICKETTSIAE

In India and the Far East typhus-like conditions occur which are frequently of a milder type than European typhus —e.g. *Japanese Flood Fever* (*Tsutsugamushi*) transmitted by a mite *Trombicula akamushi*, and due to a rickettsia designated *R. orientalis*. The so-called "*rural*" or "*scrub*" *typhus* of Malaya is a similar infection. A peculiarity of this disease is the fact that while the Weil-Felix reaction with *B. proteus* X19 is absent, a variant of this organism (the Kingsbury strain—XK) is agglutinated by the serum. Rats and probably other rodents are reservoirs of the infection.

A typhus-like condition of America, *Rocky Mountain Spotted Fever*, has been shown to be due to a species designated *Rickettsia rickettsi*. This organism is demonstrable in certain ticks—e.g. *Dermacentor andersoni* and *variabilis*—which transmit the disease. It is similar in appearance to *R. prowazeki*. This infection is passed from one generation of ticks to another. No animal reservoir of infection has yet been defined. Rickettsia bodies have been observed also in endothelial cells in the human disease. The agglutination reaction with *B. proteus* X19, X2 and XK occurs in this infection but is usually weak.

Similar infections such as "Weigl's disease" (*R. weigli*) and recurrent fevers in Poland, Russia, Japan and in Africa have been described.

Sao Paulo Typhus of Brazil is transmitted by ticks. In this condition the serum is stated to agglutinate strongly *B. proteus* X19 but this is questioned as the disease seems to be related to Rocky Mountain spotted fever. The causative organism has been designated *R. braziliensis*.

Fièvre boutoneuse of the Mediterranean is transmitted to man from dogs by a tick. The serum gives a weak reaction with *B. proteus* X19 and X2. The infecting organism is *R. conori*.

Trench Fever.—This condition was prevalent among troops during the war of 1914–18 and like typhus was louse-borne. A rickettsia (*R. quintana*) was found in lice fed on patients and has been regarded as the cause of the disease. Unlike *R. prowazeki* it does not invade the alimentary epithelium of the insect and is less pleomorphic.

The *Q fever* of Australia (*R. burneti*) is a tick-borne disease to which the bandicoot is the likely animal reservoir. There is also hereditary transmission in the tick. In this disease the serum does not agglutinate the X strains of *B. proteus*. A similar disease *X fever* of Montana has been described as being due to a rickettsia but this has not been confirmed.

The rickettsial aetiology of Trachoma and the relationship of the disease to other rickettsial infections found associated with conjunctivitis of animals are still controversial.

Heart-Water of cattle in South Africa is a tick-borne disease and a rickettsia (*R. ruminantium*) has been demonstrated in the vascular endothelium of infected animals and in the arthropod vector.

The agglutination reactions of the various rickettsial infections may be summarised as follows (after Felix).

		B. proteus.		
		X19	X2	XK
Classical typhus	Louse-borne	+++	+	—
Tabardillo	Murine type of typhus	+++	?	—
Fièvre nautique		+++	?	—
Urban typhus of Malaya		+++	+	—

(*Continued on p. 564.*)

		B. proteus.		
		X19	X2	XK
Tsutsugamushi (of Japan and Sumatra)	Mite-borne	−	−	+++
Scrub typhus of Malaya		−	−	+++
Rocky Mountain Spotted Fever	Tick-borne	+	+	+
Fièvre boutoneuse		+	+	−
South African Tick Fever		+	+	+

+ marks indicate the relative strengths of the reactions.

Note.—Occasionally in the tick-borne fevers (*e.g.* in America and South Africa) X2 is moderately or strongly agglutinated but not X19.

RICKETTSIAL AGGLUTINATION TESTS

Recently in the Middle East agglutination tests with suspensions of Rickettsiae derived respectively from classical and murine typhus have been successfully used for diagnostic purposes (van Rooyen and Bearcroft[1]) and it has been possible in this way to distinguish between these two types of the disease, whereas both yield similar results in agglutination tests with *B. proteus* X strains. Concentrated and purified rickettsial suspensions from egg yolk-sac cultures (*vide supra*) have been found suitable for the tests after dilution with a phosphate buffer mixture of pH 7·2. (Suspensions prepared by differential centrifuging from the lungs of infected mice have also been used.) The opacity of the suspension should approximate to that of Brown's standard No. 1. Serum dilutions ranging from 1 in 100 to 1 in 6,400 are made up in physiological saline in the usual way and an equal volume of the rickettsial suspension is added to each. The test includes also a suspension control without serum. Dreyer's conical agglutination tubes are very suitable for the tests. The tubes are incubated at 42° C. for four hours and then placed in the refrigerator at 0°–4° C. overnight, after which readings are made. The agglutinated Rickettsiae form very fine granular floccules and to make readings and observe accurately the end-point, the tubes are examined with a bright artificial illuminant against a dark background. Sharp rotation of the tubes assists in the recognition of the clumps. If there is any difficulty films are made from the deposit after removal of the supernatant fluid and stained by a method suitable for demonstrating Rickettsiae (*vide* p. 226). The clumps are seen as small regular masses in the film.

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[1] *Edin. Med. Jour.*, 1943, **50**. 257.

Normal serum or serum from other diseases rarely agglutinates Rickettsiae beyond a titre of 1 in 100. The reaction in typhus appears about the sixth or seventh day of the illness when the titre may be 1 in 200 ; by the eighth or ninth day the titre may rise to 1 in 400 while on the tenth to fourteenth day it reaches 1 in 800 to 1 in 6,400. The test with the two types of Rickettsiae in parallel, especially after strong reactions have developed by the tenth to fifteenth day, allows a differential diagnosis to be made by virtue of the definitely higher titre for the infecting type of Rickettsia. Thus, serum from a case of classical typhus should give a titre for the homologous type three to four times higher than that for the murine type.

BARTONELLA BACILLIFORMIS (OROYA FEVER)

Oroya fever is a disease occurring in Peru, with a high degree of mortality, characterised by intermittent fever and anaemia.

The infective agent—*Bartonella bacilliformis*—invades red blood corpuscles and is found as minute rod-shaped forms 0·3–2·5μ in length, while coccal forms also occur. It is actively motile. By Giemsa's method it stains reddish violet in colour ; the extremities appear thickened and darker in colour, and the outlines hazy. The organism is Gram-negative. Cultures are obtained in semi-solid leptospira medium and on solid or semi-solid blood media, but not in ordinary broth or agar. The optimum temperature is 25°–28° C. and the organism can survive in blood or in cultures for many weeks.

The disease can be reproduced by the intravenous inoculation of monkeys, except that anaemia does not occur. Subcutaneous inoculation into the eyebrows of a monkey also produces nodules in which numerous organisms are present.

In *Verruga peruviana*, a disease characterised by a nodular eruption, an organism closely resembling *Bartonella bacilliformis* has been isolated. Noguchi's work has proved that these organisms are serologically identical, and that Oroya fever and Verruga peruviana have the same aetiological origin.

It seems possible that the organism described as *Anaplasma* (*vide* p. 583) in an infection of cattle and found also in red corpuscles is related biologically to *Bartonella.*

Bartonella muris.—It has been observed that after splenectomy in rats, organisms of the same type as *Bartonella bacilliformis* frequently appear in the red cells and this infection is associated with marked anaemia and often produces a fatal result. The organism has been named *Bartonella muris.* Presumably, latent infection is prevalent in rats associated with a certain degree of natural immunity which is broken down by the removal of the spleen. The infection is louse-borne. A similar organism has also been noted in mice following splenectomy.

CHAPTER XXII

THE FUNGI

INFECTIONS produced by the fungi (*vide* p. 4) are usually designated "mycoses."[1]

FUNGI IMPERFECTI

The more important of the mycoses are due to organisms of the group above-named, *i.e.* fungi whose complete life-cycle is still imperfectly known, though it is possible that many of them are asexual stages of *Ascomycetes* (*vide infra*). The group includes *Microsporon*, *Trichophyton*, *Achorion*, etc..

MICROSPORON

This organism is the common cause of scalp ringworm in children. It rarely attacks the body. It is apparently unable to infect the scalp after puberty.

The mycelium develops in the horny layer of the scalp epidermis, and in the hair medulla from which hyphae pass through the cortex, and produce a covering of small spherical "spores" (approximately 5μ in diameter) arranged in the form of a mosaic on the outside of the hair. Grown artificially, *Microsporon* develops circular colonies with a raised centre and radiating folds. The surface of the colonies is velvety, due to the projecting aerial hyphae. Though the surface remains white or buff-coloured, the substance of the colony may develop a brown pigmentation.

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[1] For detailed information on this subject reference may be made to *Medical Mycology*, by Dodge, London, 1936; *Fungi and Fungous Diseases*, by A. Castellani (Adolph Gehrmann Lectures, University of Illinois College of Medicine, 1926; reprinted from *Archives of Dermat. and Syph.*, October 1927–March 1928); or *A System of Bacteriology*, Med. Research Council, 8, i.

Various species have been recognised—e.g. *M. audouini* (the common type in ringworm of the scalp among children), *M. canis* (dermatomycosis of dogs), *M. felineum* (of cats), *M. equinum* (of horses). Species of animal origin, e.g. *M. felineum*, may infect the human subject.

TRICHOPHYTON

This organism may occur in ringworm of the scalp, nails and glabrous skin. It may also be found in beard ringworm.

The fungus mycelium, as it develops in the hair medulla or epidermis, produces characteristic chains or "rosaries" of oval or rectangular "spores", which are larger (5μ or even 8μ) than those of *Microsporon*. In artificial culture the different species vary in detailed characters, but present the same general type of growth as *Microsporon* though the colonies are more dense and opaque. A common species in this country occurring in scalp ringworm is *T. crateriforme*, which produces raised colonies with crater-like depressions. Some species are markedly pigmented—e.g. *T. rosaceum*, *T. violaceum*, *T. sulphureum*.

Different species associated with dermatomycoses in animals have been recognised: *T. faviforme* (in cattle), *T. equinum* (in horses), *T. caninum* (in dogs), *T. felineum* (in cats). *Trichophyton* infections in animals are not uncommon sources of human ringworm.

ACHORION SCHÖNLEINII

Associated with the condition called "favus," which affects the scalp and other skin areas.

The fungus forms the characteristic concave yellow disks or "scutula" centred in hair follicles. The scutulum is composed of mycelium and spores, which are markedly irregular in size and arrangement.

The hairs in the affected area are also invaded. The artificial growth on culture medium consists of a tough brownish layer with raised irregular folds, and a white velvety surface due to the aerial hyphae.

Achorion quinckeanum is the species of cat and mouse favus; it is occasionally found in the human disease. A favus-like condition occurs in fowls due to *Achorion gallinae*.

EPIDERMOPHYTON

This type of organism does not attack the appendages but grows in the superficial layers of the epidermis, and skin folds are specially susceptible. The infection is exemplified by Tinea cruris (" Dhobi itch "); a similar condition may occur between the toes (Tinea interdigitalis). A number of species of *Epidermophyton* have been recognised and differentiated mainly by the colour of growths on culture medium—e.g. *E. inguinale* (yellow), *E. perneti* (pink), *E. rubrum* (deep red).

Microsporon (or *Malassezia*) *furfur* of pityriasis versicolor, *Endodermophyton concentricum* of tinea imbricata (in which the mycelial growth occurs between the rete Malpighi and stratum corneum of the epidermis), and *Endodermophyton castellanii* of tinea intersecta are further examples of " fungi imperfecti " responsible for human dermatomycoses. *M. furfur* is more of the nature of an epiphyte, growing on the skin surface, than a true infecting parasite.

A fungus, designated by some writers *Microsporon minutissimum*, is associated with "erythrasma," consisting of scaly patches in the axillae and groins with some hyperaemia. The parasite infects the epidermis and grows in the form of small threads which readily break up into shorter forms resembling bacilli. It is doubtful if this organism has been cultivated and in biological characters is more allied to the actinomycetes than to the fungi proper.

Sporotrichon beurmanni.—Associated with subcutaneous ulcerating granulomata. The condition is designated " sporotrichosis " and occurs in man and horses. When grown artificially (at room temperature) the organism forms a typical mycelium ; the hyphae are relatively narrow (2μ) ; oval spores are found in clusters at the ends of hyphae. In the tissues it may be difficult to demonstrate the organism by microscopic examination ; the morphology is entirely different, and no definite mycelium is observed ; oval or spindle-shaped forms are found ; some of these are not unlike large bacilli ; budding may be noted as in the saccharomycetes.

This organism is pathogenic to mice and white rats, experimental inoculation of cultures producing an ulcerating granuloma at the site of injection.

Hemispora stellata.—The organism of hemisporosis, a granulomatous and suppurative condition, occurring in the human subject and involving the skin in the form of single or multiple lesions. The infection may also occur in bones. This fungus, which appears as a mycelium with star-like conidiophores, is easily cultivated from lesions as an irregular whitish layer on the surface of the medium. It is pathogenic for the rabbit on experimental inoculation. It is not uncommon as a saprophyte and may be found in dust.

Allergic Reactions in Fungus Infections.—Deep-seated infections of the skin, especially those due to *Trichophyton*, may lead to widespread cutaneous hypersensitiveness to the products of the organism. Thus, in the course of the infection, generalised skin eruptions of an allergic nature may occur—*e.g.* the "trichophytides." Evidence of such allergy may be elicited by injecting extracts of the organism—*e.g.* trichophytin. These allergic reactions are not species-specific.

(In some individuals manifestations of hypersensitiveness may result from the inhalation of certain saprophytic fungi present in air or dust, *vide* p. 47 under *atopy.*)

Madurella, a fungus found in mycetoma, is referred to on p. 533.

PHYCOMYCETES

Mycelium made up of non-septate hyphae ; asexual spores developed in a spherical "sporangium" or spore-case borne on the expanded end of an aerial hypha ; "zygospores" also formed as the result of conjugation of two hyphae at their tips. These fungi are of little or no importance as pathogens, though they have been noted in auricular, naso-pharyngeal and pulmonary mycoses, and in mycosis of the tongue. They are frequently contaminants of food, and of culture medium in the laboratory. The common mould *Mucor mucedo* exemplifies the group.

Rhinosporidium sieberi.—This organism has been classified with the Phycomycetes but its biological relationships are doubtful. It is responsible for a polypoid disease of the nose and tumour-like lesions of the conjunctiva, uvula and mouth occurring in India and South America—*e.g.* Argentine. In an early phase of its life history it appears in the tissues as a small rounded capsulated body with a single nucleus. This cell enlarges and the cytoplasm and nucleus divide into a large number of "spores." The parent structure, surrounded by a thick cellulose capsule, may reach even 2–3 mm. in diameter, and with the contained spores has been regarded as analogous to a sporangium. The spores ultimately escape through a pore in the capsule and spread in the lymphatic spaces.

ASCOMYCETES

Mycelium typically septate; "ascospores" (usually eight in number) developed in a spore-case ("ascus") formed at the end of a hypha. The moulds *Aspergillus* and *Penicillium* are examples of this group.

Aspergillus is a frequent contaminant of culture media, occurring as a felted, yellow, green or black mould-growth; rows of spores or "conidia" develop from finger-like processes or "sterigmata" borne on an expanded aerial hypha. Pulmonary aspergillosis, due to *A. fumigatus*, may occur in birds—*e.g.* pigeons and penguins in captivity—and this infection has also been noted in man (among bird fanciers). *A. pictor* is responsible for a skin disease of Central America called Pinta, but other species of fungi are also associated with different varieties of this condition.

Penicillium, which includes a large number of species, is one of the commonest food moulds and contaminants of culture medium. Its biology is similar in some respects to that of aspergillus. The common green mould (*P. expansum*) found on food is a representative of this group.

Penicillium notatum.—See p. 498 and **Appendix.**

SACCHAROMYCETES

(Blastomycetes, Yeasts)

These are often classified with the *Ascomycetes*, and the yeasts are now regarded more as a phase of other types of fungi than as a separate group. Some of them have been classified among the *Fungi imperfecti*. Their morphology and gemmation, or budding, are highly characteristic features, and though in some species hyphae occur, it is convenient to consider them separately from the moulds. An outstanding biological property of these organisms is the fermentation of carbohydrates.

Pathogenic types :

Coccidioides : This genus shows yeast-like forms, but is also characterised by the formation of mycelium, and ascospore-like structures have been noted. The biological position of the genus is somewhat doubtful but it may conveniently be grouped along with the yeast-like organisms.

Coccidioides immitis—of " Blastomycosis," an uncommon granulomatous or suppurative condition involving skin, subcutaneous tissue, lungs, etc., developing sometimes into a generalised pyaemic condition.

Monilia.—This type is also yeast-like in morphology and exhibits the characteristic gemmation, but hyphal filaments also occur. No ascospores have been noted. Various carbohydrates are fermented with both acid and gas production.

M. albicans (*Oidium albicans*) is the species found in thrush (oral and vaginal). As seen in wet preparations from the lesions or in dried films stained by Gram's method, the organism appears as septate hyphae forming a mycelium along with yeast-like structures which are regarded by some as buds (blastospores) from the mycelium though they may occur as independent units, often in clusters.

M. psilosis was at one time described as the causative organism of Sprue but has probably no aetiological relationship to this disease.

M. tropicalis is the commonest type found in bronchial infections (broncho-moniliasis).

Cryptococcus : typical yeast-like forms ; no hyphae occur ; when grown in fermentable carbohydrates no gas is produced.

C. gilchristi—of Blastomycetic dermatitis.
C. linguae pilosae—of " Black-tongue."
C. farciminosus—of Epizootic Lymphangitis in horses.

Monilia and *Cryptococcus* types have been shown to be aetiological agents of dermatitis (in circumscribed form), suppurative paronychia and deformities of the nail-plate.

Torula histolytica (*Cryptococcus hominis*).—This is a yeast-like organism which reproduces only by budding, shows no mycelial formation, and produces no endospores. It has been stated to have no fermentative properties, but some strains resemble the cryptococci in fermenting carbohydrates without gas production. It seems doubtful whether *Torula histolytica* can be separated from the *Cryptococcus* group. It has been reported in cases of meningitis and also in pulmonary infections. Mice are susceptible to inoculation, *e.g.* intraperitoneally, and develop a general infection with granulomatous lesions in internal organs and muscles.

Histoplasma capsulatum.—This organism has been described in a tropical disease characterised by splenomegaly and anaemia. It was originally thought to be a protozoon but its fungus nature has been clearly established. In the tissues yeast-like forms are seen, and mycelium is developed in culture. The organism has recently been classified with the *Monilia* group.

DIAGNOSIS OF THE DERMATOMYCOSES

The affected hairs, or the epithelial scales from affected areas, are treated on a microscope slide with a 10 per cent. solution of caustic potash, gently warmed, and examined (with a $\frac{1}{6}$-in. lens) in the unstained condition under a cover-slip. The alkali clears the hairs and epidermis, and renders the fungi easily detectable under the microscope. Artefacts that may be mistaken by inexperienced observers for fungal elements, and therefore to be remembered in diagnostic work, are oil or fat droplets, air bubbles, spaces between epithelial cells, etc.. Stained preparations are unnecessary for routine diagnostic work.

In examining scales from the palms or soles in cases of suspected epidermophyton infection "mosaic" or "ghost" fungus is frequently seen, *i.e.* rectangular spore-like structures between epithelial cells. It is now known that this appearance is not due to a fungus but to cholesterol crystals between the cells.

For artificial culture, Sabouraud's medium, standardised to pH 5·0—5·5 (*vide* p. 154), is suitable, and it tends to restrain the growth of bacteria. The original procedure was to solidify a layer of the medium in the bottom of a conical flask. After inoculation the mouth of the flask was covered with a rubber cap over the cotton-wool plug, or sealed with paraffin (*vide* p. 172). A more convenient arrangement is to use as the container a screw-capped vial or medical flat bottle and to solidify the medium with the bottle in the horizontal position. This provides sufficient area of medium for inoculation and growth of the fungus, and the morphological characters of the growth can easily be observed. The screw-cap prevents evaporation. In making cultures from scales and hairs, two minutes' immersion in 95 per cent. alcohol before "planting" them on the medium assists in the isolation of the fungus by destroying the cocci and other bacteria that may be present on the skin. The addition to the medium of 1 : 500,000 (0·0002 per cent.) gentian violet or 1 : 200,000 brilliant green assists in the isolation of the fungus by inhibiting contaminant bacteria. (The pH should be adjusted to 5·5 before sterilisation.) The cultures are incubated at 25° C.. The growth of the fungus may be relatively slow, and the cultures may have to be incubated over a prolonged period to elicit characteristic appearances. It is for this reason that the containers must be sealed to prevent drying of the medium.

The tellurite-trypsin-agar as used for the isolation of the diphtheria bacillus (p. 141) is also suitable for the growth of the ringworm fungi. The medium is inhibitory to skin organisms such as staphylococci and it enables pure growths to be obtained from primary cultures. The ringworm hairs are treated with 50 per cent. alcohol for five minutes, and then placed on the surface of the medium in Petri dishes. Growths visible with a hand lens can be obtained after three to five days' incubation, while growths visible to the naked eye can be seen in a week to ten days (Allison).

CHAPTER XXIII

MALARIA PLASMODIA; BABESIAE; TRYPANOSOMES; LEISHMANIAE

THE MALARIA PLASMODIA

MALARIA is a protozoal disease in which the causative organism—*Plasmodium*—invades the red cells of the blood. Three well-defined species of the malaria plasmodium are recognised :

P. vivax—Benign tertian malaria.
P. malariae—Quartan malaria.
P. falciparum (*Laverania malariae*) — Malignant malaria.

These organisms belong to the order *Haemosporidia* of the *Sporozoa*. The plasmodium is transmitted by anopheline mosquitoes, and goes through the sexual phase of its life-cycle in the body of the insect. Only the asexual stage is completed in the human subject.

Outline of the life history of the Malaria Plasmodium. —It is introduced into the body by the bite of the mosquito (*vide infra*) as a minute spindle-shaped cell or *sporozoite*, containing nuclear material in the form of a granule of chromatin. The sporozoite enters, or becomes attached to, a red cell, and assumes an amoeboid form which gradually develops (*trophozoite*). It grows at the expense of the red cell and accumulates altered blood pigment in the form of brownish granules. It may display amoeboid movement and protrude pseudopodia. More than one trophozoite may attack a single corpuscle. The corpuscle may show considerable alteration in size as the trophozoite develops.

The trophozoite tends to assume a characteristic ring-form due to the formation of a vacuole-like structure, with the chromatin granule or " dot " at one side, so that the whole organism resembles a signet-ring. When full-grown it is more or less rounded, and may appear to occupy the greater part of the corpuscle. These *mature trophozoites* usually contain a considerable amount of blood pigment.

When fully developed, the trophozoite becomes a *schizont*, and *schizogony* takes place. The pigment accumulates towards the centre, the chromatin breaks up into smaller particles, the protoplasm subdivides and forms a number of small round or oval *merozoites* (about 2μ in diameter) each containing a fragment of the original chromatin. The residual protoplasm and pigment remain in the centre of the group of merozoites. The number of merozoites resulting from schizogony varies with the different species (*vide infra*). Finally the individual merozoites are liberated as free structures in the blood. The merozoite attacks another red cell and thus the asexual cycle is repeated. The pigment is taken up by leucocytes.

The length of time the organism takes to complete the asexual cycle varies with the species :

P. vivax	Two days.	Dltian
P. malariae . . .	Three days.	Terth
P. falciparum . . .	One or two days.	Fa

The fever develops at the stage of schizogony ; thus in *P. vivax* infections the febrile paroxysm occurs every second day (tertian malaria), in *P. malariae* infections every third day (quartan malaria).[1]

In malignant malaria the parasite leaves the peripheral blood before it becomes a mature trophozoite

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[1] The terms " tertian " and " quartan " are derived from the occurrence of the successive attacks on the third and fourth days respectively, counting the day of the previous attack as the first.

DIFFERENTIATION OF THE MALARIA PLASMODIA

	P. Vivax (*Benign tertian*)	P. Malariae (*Quartan*)	P. Falciparum (*Malignant*)
Asexual life-cycle	48 hours	72 hours	24 to 48 hours
Trophozoites in fresh unstained preparation of blood . .	Not refractile, hyaline, not easily observed; usually one organism only in red cell; active movement	Refractile, "frosted-glass" appearance; more easily observed; not amoeboid and active	Small, about ⅛th or ⅙th of diameter of red cell; often more than one organism in cell; active at first
Pigment in trophozoites .	Fine, yellowish-brown, and evenly distributed	Coarse, brownish-black	Scanty, fine
Ring forms (stained preparations) .	Large, irregular, not well defined, about ⅓rd diameter of red cell; usually single chromatin dot	Thick round rings, about ⅓rd diameter of red cell; often in equatorial bands	Small, well defined, thin; often 2 chromatin dots; often situated at the edge of red cell; about ⅛th or ⅙th diameter of red cell
Red cells (stained preparations) .	Swollen, pale, showing deeply stained points, "Schüffner's dots"	Not altered	Shrivelled, deeper colour, but may be swollen and pale; may have a few cleft-like irregular dots (Maurer's dots)
chizonts (stained preparations) .	Large, mulberry-like; about same size as red cell; 12–24 oval merozoites	Small, "daisy-head"-like; smaller than red cell; 6–10 round merozoites	Small; segmentation irregular; 10–30 (usually 12) very small merozoites; rarely seen in peripheral blood
Gametocytes .	Rounded; macrogametocyte about one and a half times the size of red cell	Rounded; about the same size as red cell or smaller	Elongated, crescentic or sausage-shaped

and schizogony occurs as a rule only in the blood of internal organs.

While some of the organisms develop into schizonts, others become *gametocytes*. In the case of *P. vivax* and *P. malariae*, these are rounded and about the same size as a mature trophozoite. The gametocytes of *P. falciparum* are sausage-shaped or crescentic, with the envelope of the corpuscles stretched across the poles of the crescent. Male and female gametocytes are distinguished, and designated respectively *micro-* and *macro-gametocytes*. The micro-gametocyte, as compared with the macro-gametocyte, contains a nuclear structure which is relatively large and disposed across the body in the form of a spindle. The pigment is diffuse. In the macro-gametocyte the nucleus is small, compact and peripheral in situation. In the female gametocyte of *P. falciparum* the pigment is usually accumulated in the centre of the organism.

These gametocytes remain unchanged in the blood until it is withdrawn from the body—*e.g.* by the mosquito or when a drop is exposed to air and transferred to a warm stage for microscopic observation. In the stomach of the mosquito the following changes occur. The gametocytes of the crescent type become rounded. Both male and female gametocytes undergo " maturation " by the formation of one or two " polar bodies " which contain part of the original nuclear chromatin and are protruded and detached. The mature macro-gametocyte constitutes the *macro-gamete*. From the male cell 4 to 8 flagella-like structures are quickly protruded (" exflagellation ") ; these are long slender processes with somewhat enlarged free ends, each containing a chromatin granule derived from the parent nucleus, and are the *micro-gametes*; they are ultimately detached, and move with a kind of lashing motility. A micro- and macro-gamete unite and form the *zygote* which develops movement (*oökinete*), slightly elongates and penetrates the stomach wall, embedding itself between the muscle fibres; it rounds itself later, forms an encysting membrane (*oöcyst*) and increases in size until it projects into the body cavity; division into rounded *sporoblasts* (or *sporoblastoids*) occurs, and these divide again into the spindle-shaped *sporozoites*, thousands of which are formed from the original oöcyst; the cyst ultimately ruptures and the sporozoites are set free in the body cavity and settle in the salivary gland, from which they are injected with the salivary secretion when the insect bites. This phase in the life history is that of *sporogony* as contrasted with the asexual schizogony in the human subject; it takes seven to ten days (under favourable temperature conditions).

Plasmodium ovale (Stephens).—This is a fourth species or sub-species of malaria plasmodium, which has been reported in a few cases infected in East and West Africa. It resembles *P. malariae* but the erythrocytes are often markedly enlarged, oval in shape, show an irregular outline and exhibit to a marked degree the stippling (Schüffner's dots) as seen in the benign tertian form of malaria. The illness is tertian in periodicity. The infection has been transmitted experimentally by the bite of *Anopheles maculipennis*.

Certain other supposed sub-species of *Plasmodium* have also been described.

DIAGNOSIS

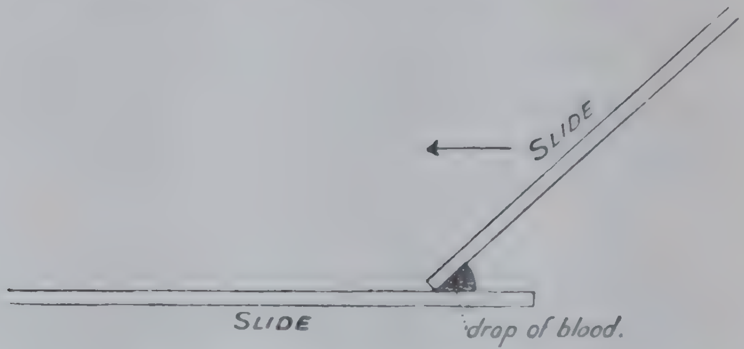
THIN BLOOD FILMS.—Two or three films are made on microscope slides or $\frac{3}{4}$-in. square No. 1 cover-slips which have been carefully cleansed (*vide* p. 191) and polished with a smooth cloth.

Requisites.—Straight triangular needle, slides or cover-slips, spirit lamp or Bunsen, gauze, methylated spirit.

The blood is obtained by puncturing the lobe of the ear, or the finger close to the base of the nail. The needle and the area to be punctured are cleansed with spirit.

Films on slides.—Touch the exuding drop of blood with the surface of a slide close to one end; place the narrow edge of a second slide kept at an angle of 45° on the drop of blood and allow the blood to spread out across the slide in this angle before drawing out the film; now spread the blood uniformly on the slide in the form of a thin film (see diagram).

Films on cover-slips.—Touch the drop of blood with the surface of a cover-slip held by the edges between the thumb and first finger of one hand, and place a second cover-slip over the first so that the drop spreads out between them. Then at once take the second slip by the edges between the thumb and forefinger of the other hand, and slide the two apart without exerting pressure.



The films are allowed to dry, and are then fixed and stained with Leishman's stain (*vide* p. 217). They are mounted and then examined, first with a dry $\frac{1}{6}$-in. lens, and later with the oil-immersion objective. In searching for crescents it is advisable to use the former. The $\frac{1}{7}$-in. oil-immersion lens (*vide* p. 72) is also very useful for this purpose.

It is essential that the film should be well stained. otherwise it is useless searching for the parasite. A valuable guide is the staining of the leucocytes in the film—if this is satisfactory malaria parasites should be detectable.

THICK BLOOD FILMS.—As a routine measure, and especially when the organisms are likely to be scanty, thick films should be prepared and examined. A large drop of blood is deposited on a slide, and spread with the head of a pin in the form of a *thick* film about $\frac{3}{4}$ in. in diameter; it is thoroughly dried—*e.g.* in the incubator—and the haemoglobin is removed by treating with acid-alcohol (alcohol 50 c.c., hydrochloric acid 10 drops) or a mixture of 4 parts of 2·5 per cent. glacial acetic acid in distilled water and 1 part of 2 per cent. crystalline tartaric acid in distilled water, and then washing in water; the film is stained by Leishman's method. See **Appendix**—Field's method.

FRESH PREPARATIONS OF BLOOD FOR MICROSCOPIC EXAMINATION.—A drop of blood is deposited on a slide, covered with a cover-slip, and the edges of the glass are smeared with vaseline to prevent drying of the film. A warm-stage apparatus should be used during the microscopic examination.

Only stained films are examined as a rule in routine work. They should, if possible, be taken during the pyrexia. The organisms can be recognised by their various characteristic appearances, and it may be possible to determine the species or type present (*vide* Table, p. 577). It must be remembered, however, that the young trophozoites of the three types may be almost indistinguishable from one another and if only young forms are present in the film, it may be difficult to determine the species. To inexperienced workers, artificial appearances may sometimes simulate malaria parasites, and a blood platelet overlying a red corpuscle may be mistaken for a young form of the plasmodium. In certain cases prolonged search may be required. It is advisable, in searching thin films for scanty malaria organisms, to examine particularly the edges of the film. They may be more numerous there than in the centre. The thick-film method greatly facilitates the detection of the parasite. The absence of parasites during an apyrexial interval by no means excludes malaria.

Certain *cytological features of the blood* may, in the absence of actual organisms, suggest the existence of a malarial infection —*e.g.* a relatively high percentage of large mononuclear cells (15–20 per cent.), or the presence of leucocytes containing altered blood pigment (*vide supra*).

Cultivation of the Malaria Parasite.—This may be utilised for diagnostic purposes, and the following method is recommended by Knowles. 5 c.c. of blood are withdrawn by vein puncture. The interior of the syringe before it is used must be thoroughly washed with saline solution. The blood is placed in a sterile flask with glass beads and defibrinated. For the actual culture, stoppered sterilised tubes, 12·5 by 1·25 cm., are employed, and in each of these is placed one drop of sterilised 50 per cent. pure glucose; defibrinated blood to a depth of 2·5 cm. is then added. Partial anaerobic conditions are produced by warming the upper part of the tube and then attaching a rubber teat to the mouth. The tubes are incubated at 37° C.. After twelve hours, and also after twenty-four or forty-eight hours, a small amount of the surface layer of the deposited red cells is aspirated with a capillary pipette and from this films are made on slides and stained by the usual method.

PIROPLASMS (or BABESIAE)

These protozoal organisms (classified with the Sporozoa) produce disease in various domesticated animals, but are not known to infect the human subject.

They invade red blood corpuscles like the malaria plasmodia, and multiple infection of these cells is characteristic. The individual organisms are pyriform bodies about 2μ to 4μ in length, containing a well-defined chromatin structure. The central part of the organism often stains less deeply than the periphery, and ring-forms like those of the malaria parasite may be observed. Some species show small rod-shaped forms. Multiplication occurs by binary fission and pairs of individuals partially attached to one another may be seen inside the red cells. For microscopic demonstration, blood films are stained by a Romanowsky stain—*e.g.* Leishman's or Giemsa's. These organisms are usually transmitted by ticks, and the eggs may also become infected. For detailed information regarding the biology and life-cycle of these organisms, one of the works on protozoology should be consulted.

Babesia bigemina is the causative organism of Red Water Fever of cattle in North and South America, Africa and Australia. It is transmitted by ticks (e.g. *Margaropus annul-*

atus). It occurs in characteristic pairs in the red cells. The individuals are pear-shaped with the pointed ends in apposition.

Babesia canis is the organism of Biliary Fever of dogs. In the blood the babesiae are seen both in the red cells and plasma. The intracellular forms are pear-shaped or irregular, and several may be found in one corpuscle. The free forms are rounded.

Organisms resembling *Babesia bigemina* occur in "red water fever" of cattle in Europe (*Babesia bovis*), Biliary Fever of horses (*Babesia equi*) and haemoglobinuria of sheep (*Babesia ovis*).

Babesia (or *Theileria*) *parva* of East African Coast Fever in cattle appears as a small rod-shaped body inside red cells. Ring-forms may also be observed. *Babesia* (or *Theileria*) *mutans* is a somewhat similar organism producing a relatively mild infection of cattle in South Africa.

A genus designated *Anaplasma*, originally thought to be related to *Babesia*, has been described in Gall Sickness of cattle in South Africa. The organisms, as seen in blood films stained by Giemsa's stain, appear as minute rounded bodies situated near the margin of the red cell (*A. marginale*), in size about one-tenth of the diameter of the cell. They are described as possessing a central granule. These structures may possibly be related to *Bartonella* (*vide* p. 565).

TRYPANOSOMES

Protozoa of the class Mastigophora. Infection with these organisms is designated by the general term Trypanosomiasis.

Three species which are pathogenic to man have been described: *T. gambiense* and *T. rhodesiense* of African Sleeping Sickness, and *T. cruzi* of Brazilian trypanosomiasis. It is doubtful whether the first two are separate species. The last-named is sometimes classified in a separate genus, *Schizotrypanum*.

TRYPANOSOMA GAMBIENSE

Biological characters.—This organism is an elongated, sinuous, fusiform structure, 8–30μ long by 1·5–3μ broad, with a longitudinal undulating membrane and a flagellum projecting from one end. It is motile, and in moving the flagellum is anterior. In stained preparations two nuclear structures are noted, the larger or trophonucleus situated about the middle of the organism, and the smaller micro- or kineto-nucleus (or kinetoplast) at the posterior end. The latter stains deeply and is surrounded by an unstained halo. Two constituent structures have been distinguished in the micronucleus: a granule (blepharoplast) from which the axoneme arises (*vide infra*) and the "parabasal" body. Chromatin-like granules are seen in the protoplasm independently of the nuclei. From the blepharoplast arises a filament, the axoneme, which forms the free edge of the undulating membrane and is continued into the flagellum, forming its central core. This structure stains like chromatin. Morphological variation (polymorphism) is noted among individual organisms, some being relatively long and slender with long " free " flagella, others broader and with a short flagellum or lacking a free flagellum. Intermediate forms are also observed.

Multiplication is by longitudinal amitotic fission.

Occurrence.—The infection is transmitted by the bite of *Glossina palpalis* (*Tsetse fly*) and in certain areas by *G. tachinoides* and *G. morsitans*. Shortly after blood from an infected person is ingested by the insect, transmission is possible in a mechanical fashion. A later stage of infectivity occurs after about twenty days; the trypanosomes have multiplied in the intestine and passed to the proventriculus, the salivary glands and proboscis. Multiplying in the insect, the organism may show considerable change of form. Thus the undulating membrane may be lost, the kinetonucleus becomes situated just in front of the macronucleus, and a free flagellum may or may not be present (*crithidial form*). From these crithidia in the salivary gland the so-called " metacyclic " forms develop, *i.e.* resembling the short and broad trypanosomes seen in the blood (*vide supra*). The fly does not become infective until the metacyclic forms are present in the salivary gland.

T. gambiense occurs also in wild herbivora—*e.g.* certain antelopes—from which it is transmitted by tsetse flies.

Two or three weeks after infection a febrile condition develops, and trypanosomes are present in the blood, though not as a rule sufficiently numerous to be readily demonstrable. The superficial lymph glands, *e.g.* posterior cervical, become enlarged, and trypanosomes can be demonstrated by puncture and aspiration with a syringe. In the advanced stages of the disease, when the characteristic lethargy has developed, the parasites can be detected in the cerebrospinal fluid.

Animal Inoculation.—Monkeys injected with infective material develop a disease which is more or less similar to human trypanosomiasis.

Guinea - pigs can be infected, and trypanosomes appear in considerable numbers in the blood, but the infection is either unassociated with any obvious pathological condition, or the resulting disease is very chronic in its course.

Cultivation of Trypanosomes.—See p. 152.

TRYPANOSOMA RHODESIENSE

Associated often with a more acute form of Sleeping Sickness than that produced by *T. gambiense.*

Glossina morsitans is the insect vector.

Morphologically it resembles *T. gambiense*, but, *in an inoculated animal*, a certain number of the trypanosomes (about 5 per cent.) show the trophonucleus situated posteriorly near the kinetonucleus (" posterior nucleated " forms).

This organism is also stated to be more virulent to laboratory animals. The question whether or not it represents a separate species is still unsettled, and it has been pointed out that *T. gambiense* may also exhibit posterior nucleated forms in inoculated animals.

It has been supposed that this organism may be identical with *T. brucei*, the organism of Nagana, but it has been shown by inoculation of man that *T. brucei* is not pathogenic to the human subject.

TRYPANOSOMA (or SCHIZOTRYPANUM) CRUZI

The cause of human trypanosomiasis in Brazil (Chagas' disease).

Its first development after infection occurs in the endothelial and tissue cells of internal organs, in the muscles and in the heart wall. It is non-flagellate at first, and resembles *Leishmania* (*vide infra*). It may, however, appear in the blood as a typical flagellate trypanosome with a very prominent kinetonucleus.

Certain other vertebrate hosts harbour the organism—especially the armadillo.

It is transmitted by bugs—e.g. *Panstrongylus megistus* (syn. *Conorhinus* or *Triatoma megistus*).

Various laboratory animals are susceptible to experimental inoculation—*e.g.* guinea-pigs, white rats, monkeys.

DIAGNOSIS OF TRYPANOSOME INFECTION

In the first place the peripheral blood should be examined. As trypanosomes may be scanty, "thick films" are prepared and stained by Leishman's stain as in malaria diagnosis (*q.v.*). Fresh preparations of the blood may also be examined microscopically (*vide p.* 581).

A method of concentrating trypanosomes in the blood has been applied as follows: 5–10 c.c. of blood are withdrawn from the vein into 20 c.c. of 1 per cent. sodium citrate solution, and the mixture is centrifuged for about ten minutes; the plasma and leucocyte layer on the surface of the blood sediment are withdrawn and re-centrifuged; this is repeated two or three times; the deposit is examined, in the form of fresh preparations and stained films, after each centrifuging.

If superficial lymph glands are enlarged, puncture and aspiration with a syringe may be carried out, and the "juice" examined. The syringe should be perfectly dry if this procedure is to be successful.

Blood, gland juice or an emulsion of an excised gland injected into a guinea-pig may yield a positive diagnosis where other methods fail. The blood of the animal is examined in fresh preparations or stained films at intervals after the inoculation.

In the lethargic state, 10 c.c. of cerebro-spinal fluid are withdrawn, and centrifuged for fifteen to twenty minutes; the deposit is then examined either in the form of a fresh preparation under a cover-slip ringed with vaseline, or in stained films.

Auto-agglutination of blood cells is frequently observed in trypanosomiasis and is a useful indication for diagnostic purposes. A simple method for detecting this phenomenon is to spread a drop of blood on a slide under a cover-slip and examine microscopically. The cells instead of forming rouleaux become aggregated in compact clumps. The reaction is probably of the same nature as the "cold agglutination" observed in primary atypical pneumonia (p. 636).

TRYPANOSOMIASIS OF ANIMALS

Trypanosome infections occur in a variety of animals. Different species of trypanosomes are recognised. These have the general characters of the genus as described in the case of *T. gambiense*, but show variations in certain of their characters.

T. brucei: the organism of Nagana or Tsetse Fly disease occurring in horses, other equidae, dogs and cattle in Africa, is transmitted by *Glossina morsitans* and certain other species of *Glossina*. It is sluggish in movement and corresponds in its morphology with *T. rhodesiense* (*vide supra*), showing posterior nucleated forms.

T. vivax, so called in virtue of its active movement, and *T. congolense* produce infections in domesticated animals in Africa and are transmitted by tsetse flies. *T. vivax* possesses a "free" flagellum. *T. congolense* is a smaller organism (9–18μ long) and has no "free" flagellum.

T. equinum is the organism of Mal de Caderas, a South American disease of horses. A feature of the organism is the inconspicuous kinetonucleus which stains feebly.

T. evansi : the organism of Surra affecting domesticated animals (including camels) in various parts of the world. It is actively motile, with a " free " flagellum and a blunt posterior end. It is conveyed mechanically by *Stomoxys* and *Tabanus* flies.

T. equiperdum occurs in horses, producing the disease known as Dourine ; the infection is transmitted by coitus. The organism shows a " free " flagellum and is very similar to *T. evansi.*

T. lewisi is an exceedingly common blood parasite of rats, and is world-wide in distribution. It is an actively motile, narrow trypanosome, with a pointed posterior end and anteriorly a " free " flagellum. The kinetonucleus is rod-shaped and stains deeply. It produces little disturbance in the health of the host. Infection is transmitted by rat fleas.

T. theileri occurs in cattle in South Africa. It is unusually large (25–70μ in length). The flagellum is " free." It is possibly transmitted by a Tabanid fly. The pathogenicity of this organism is doubtful.

For further details of the pathogenic trypanosomes one of the larger works on bacteriology or protozoology should be consulted.

LEISHMANIAE

These are pathogenic protozoa biologically related to the trypanosomes.

They are designated as follows :

L. donovani—of Kala-azar (occurring in certain Eastern countries).

L. tropica—of Tropical sore or Delhi boil. This is generally regarded as a separate species.

" *L. infantum* "—of Infantile splenomegaly (occurring in Northern Africa). It is doubtful whether this form constitutes a species separate from *L. donovani.*

" *L. braziliensis* "—of Espundia (or Uta), occurring in South America. This disease resembles in some respects Tropical sore but affects specially the nasal and pharyngeal mucous membranes.

LEISHMANIA DONOVANI

In morphology it is a round or oval organism about 2–5μ in its longest diameter.

Stained with a Romanowsky stain two nuclear structures are observed, one large and rounded (macronucleus), and the other small, deeply staining, and rod-shaped (kinetoplast). As in the trypanosomes this latter structure consists of a parabasal body and a prolongation corresponding to the axoneme (rhizoplast). The protoplasm may be vacuolated. The organism multiplies by binary fission.

The *Leishmania* is typically intracellular in the tissues, situated in the endothelial cells of the spleen, liver, bone marrow and lymphatic glands. It may also be found in large mononuclear cells in the peripheral blood. One endothelial cell may contain a considerable number of organisms.

Cultures can be obtained from the spleen on N.N.N. medium (*vide* p. 152) incubated at 20°–24° C.. In culture the organisms increase in size and elongate; the kinetoplast becomes situated at one end, and from it a flagellum arises. No undulating membrane develops. Thus, *Leishmania* in culture assumes the biological characters of a *Leptomonas*.

Monkeys and dogs can be infected experimentally. The Chinese hamster (*Cricetulus griseus*) is susceptible to inoculation and has been used for experimental studies.

Transmission of the disease is by insect agency. The evidence points to the sand-fly, *Phlebotomus argentipes*, as the vector of infection.

Diagnosis

Thick blood films should be examined as in malaria diagnosis, the organisms being searched for in large mononuclear cells; but in a large proportion of cases it may be impossible to demonstrate leishmaniae in the peripheral blood.

During life the diagnosis may be established by aspirating fluid from the enlarged spleen by "spleen puncture." A dry needle should be used. This procedure is not without danger to the patient, and for this reason liver or sternal puncture has been preferred, though the likelihood of finding the organism is less than in the case of the spleen.

A diagnosis may frequently be established by cultivating peripheral blood on N.N.N. medium.

Another diagnostic method is to inoculate Chinese hamsters with 1 to 5 c.c. of blood or material obtained by spleen, liver or sternal punctures.

The *aldehyde reaction* has also been used in the diagnosis of the disease. For this purpose about 5 c.c. of blood is withdrawn and allowed to clot. The serum is separated and to 1 c.c. are added 2 drops of commercial formalin. A positive reaction is indicated by an immediate opacity, followed within 30 minutes by the development of a firm white gelatinous coagulum (like boiled egg albumin); mere jellification is not accepted as a positive result.

LEISHMANIA TROPICA

This organism is similar to *L. donovani* and shows the same intracellular distribution. Besides the characteristic oval forms, elongated organisms may be noted. In culture on N.N.N. medium the leptomonas forms develop as in the case of *L. donovani*. Monkeys and dogs can be infected experimentally. Transmission is probably by *Phlebotomus papatasii* and *Phlebotomus sergenti*.

Diagnosis.—Films are made from the exudate of the sore (preferably at the margin) after carefully cleansing the surface and removing the surface discharge. They are stained by Leishman's stain.

"LEISHMANIA INFANTUM"

This organism is similar to *L. donovani*, and is associated with a pathological condition, similar to that of Kala-azar, occurring in young children in North Africa and the Mediterranean littoral. The infection also occurs naturally in dogs, which are possibly the origin of the human disease, the organism being transmitted by an insect (probably *Phlebotomus perniciosus*). Monkeys, dogs, guinea-pigs and certain hamsters are susceptible to experimental infection.

Diagnosis is established as in Kala-azar.

CHAPTER XXIV

FILTERABLE VIRUSES[1]

CONSIDERABLE attention has been paid in recent years to the group of infective agents producing disease in man, animals and plants, which, on account of their minute size, can pass through the pores of earthenware and collodion filters capable of arresting the bacteria.

The question has been much debated whether the filterable viruses are living organisms. All the evidence at present available, microscopical and experimental, shows that these infective agents, as they affect man and animals, are organismal entities comparable with, though very much smaller than, the ordinary microbes, and alternative explanations are difficult of acceptance. The smallest viruses of animals, however, approximate in size to that of the molecules of the more complex proteins and it has been found that the infective agents of certain virus diseases of plants, *e.g.* tobacco mosaic disease, can be isolated in the form of a crystalline protein of high molecular weight. Such agents are nevertheless self-reproducing like true organisms and transmissible from one individual host to another like the pathogenic microbes, setting up in each case as a rule a characteristic disease. Whatever the true biological nature of the filterable viruses may be, they behave like parasitic microbes and their effects are studied by methods similar to those applicable in bacterial diseases.

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[1] For fuller information see *Virus Diseases of Man*, by C. E. van Rooyen and A. J. Rhodes, 1940, Oxford University Press, London.

Three characteristics have been cited in respect of which viruses differ from bacteria: (*a*) ultramicroscopic size, (*b*) filterability, and (*c*) non-cultivability on inanimate substrates. But the "elementary" particles or bodies seen microscopically in many virus diseases (*vide infra*) actually represent the causative viruses, and virus bodies have been photographed by ultra-violet light and by the electron microscope (*v. infra*). Moreover, organisms which cannot be classed with the viruses may pass earthenware filters. The terms ultramicroscopic and filterable are, therefore, not applicable to the whole series of viruses. In the present state of our knowledge and technique their non-cultivability on inanimate food materials constitutes a more distinct difference between them and the bacteria. Many viruses, however, can be cultivated along with surviving tissue cells, in tissue cultures or in the embryo-chick, and it is possible that when their biological requirements are better known, simpler methods may possibly be utilised. These facts will be elaborated later, but, at the outset, it should not be assumed that there is a distinct biological separation of the group of viruses and the ordinary micro-organisms.

As explained on p. 61, the limit of microscopic resolution for individual particles is about 0·25μ when direct illumination by transmitted light is used, and is governed by the wave-length of the light employed. By dark-ground illumination, however (*q.v.*), a very minute object may be rendered easily visible owing to its refraction of light, and is seen as a luminous spot. No resolution is obtained; that is, no details of its form or structure are revealed, but its presence merely is noted. The shorter the wave-length of the light used the smaller the object that can be resolved. Thus, if short-wave ultra-violet light be used, objects smaller than 0·25μ can be resolved, but as the eye is insensitive to such short waves photographic methods must be employed. For this work a special microscope having an optical system constructed entirely of quartz (since glass absorbs ultra-violet rays) must be employed while the technical difficulties are great. Under these circumstances Barnard has succeeded in resolving and photographing objects 0·075μ in diameter. These facts also illustrate the inapplicability of the term "ultra-microscopic." Moreover, as already pointed out (p. 61), actual visibility with the ordinary microscope may extend beyond the limit of resolution ; thus, it is possible to see stained particles as small as 0·074μ in diameter with white light by using at the correct tube length an apochromatic objective of N.A. 1·40, a high-power compensating ocular, and an oil-immersion condenser, the whole optical system being accurately centred.

In addition, while a virus in the natural state may be below the limit of visibility, after staining it may be of sufficient size to be seen as a result of the deposition of stain on the surface of the particle.

By means of the electron microscope (p. 659) photographs of virus bodies can now be obtained at very high magnification.

The evidence regarding viruses which has accumulated during the last few years indicates that those related to animal diseases are definitely living pathogenic entities comparable with the bacteria, but very much smaller in size. They propagate, infect tissues and incite immunity like bacteria, and there is no reason why the range of micro-organisms in nature should be bounded by the limit of human vision with ordinary light. Just as the visible pathogenic organisms range in size from that of the anthrax bacillus to the influenza bacillus and the pleuro-pneumonia organism, so also viruses vary considerably and some are not much larger than a molecule of the more complex proteins (*vide* p. 597).

It has been suggested that the viruses are the end-result of a retrograde evolution from ordinary microbial forms to an extreme state of parasitism in which most of the functions of the independent living organism have been lost, along with the corresponding cell substance, and only a vestige remains. Such entities on the basis of this assumption are unable to synthesise substances essential for cell growth and can only function as living organisms by obtaining these from the cells of their hosts. In this way also is explained their intracellular habitat in the tissues. It is thought that, while the larger viruses may still retain some of the enzyme systems necessary for vital activity, in the descending scale of size of these infective agents there is loss of more and more such functions, so that the smallest viruses are devoid of all the enzyme systems necessary for the synthetic activities characteristic of a living organism, and may constitute merely protein macromolecules, though these still represent the elements necessary for reproduction and maintenance of species. In the appropriate cell, however, the virus obtains the substances necessary for its growth and becomes to all intents and purposes a living organism (*vide* p. 94).

A number of viruses have now been photographed by ultra-violet or electron microscopy, or demonstrated by ordinary microscopy in the form of elementary bodies, while the approximate sizes of the virus bodies can be determined by filtration through graded collodion membranes and by their rate of deposition when centrifuged at high speeds (*vide infra*). Immune serum shows specific inactivating properties towards the virus concerned, and in certain cases such as herpes and vaccinia the antibodies can be absorbed by suitable virus material. Comparable with the serological types of bacteria, such as the pneumococcus, are the three antigenically distinct types of the foot-and-mouth disease virus termed O, A and C, and the different types of the influenza virus. Moreover, the viruses when transmitted by animal passage, in the same or different species, "breed" true to type and reproduce the same disease.

The bacteriophages, which will be dealt with later, present a close analogy to the viruses and may reasonably be regarded as bearing a parasitic relationship to bacteria like that of the viruses to the tissues of animals and plants.

The position of the *Rickettsia* group which has been described in the previous chapter and its relationship to the viruses require consideration. Certain writers actually include these organisms in the group of viruses and the relationship between them and some of the larger viruses is apparently a close one. The rickettsiae like the viruses also require living tissue cells for their growth. Until the biology of all these infective agents is better known it is perhaps expedient to classify the rickettsiae separately.

SIZES OF THE VIRUS BODIES

By filtration through thin collodion membranes (Elford), and by high-speed centrifugation viruses have been graded according to size.

The sizes of many of these viruses, ascertained by electron microscope photography, correspond very closely with those estimated by filtration.

The Table on page 597 indicates the estimated sizes of various filterable viruses. Owing to their minuteness the unit mμ (millimicron) is used, *i.e.* one-thousandth part of a μ, or 0·000001 mm.

As indicated above, the sizes of virus bodies have been estimated by centrifuging at very high speeds (60,000 r.p.m.), the virus being retained by a disk of thick filter paper at the bottom of the container. The rate of sedimentation follows a fixed law (Stokes' law) depending on the diameter of the particle, its density, the viscosity and density of the medium, the height of the liquid column, the distance of the filter paper from the axis of rotation, and the speed in r.p.m. The values of the factors enumerated above can be determined and the size of the particles calculated. By this means, which entails high technical skill and elaborate apparatus, the sizes of most of the different virus bodies have been computed, and they agree closely with the sizes estimated by filtration experiments and by photography with the electron microscope.

Virus, etc.	*Size in* $m\mu$
Staphylococcus (for comparison)	1000
B. prodigiosus ,,	750
Rickettsia bodies ,,	300
Psittacosis	220–330
Molluscum contagiosum	250
Smallpox	225
Vaccinia	
Canary-pox	225
Inclusion conjunctivitis	About 200
Pleuropneumonia organism (granular form)	150
Filter-passing organisms of sewage (Laidlaw and Elford)	150
Rabbit fibroma (Shope)	150
Lymphogranuloma inguinale	125–175
Infectious ectromelia of mice	125
Herpes febrilis	About 120 (100–150)
Rabies	100–150
Pseudo-rabies	100–150
Borna disease of horses	100
Influenza	80–120
Rous sarcoma of fowls	75
Vesicular stomatitis of horses	70–100
Bacteriophage, staphylococcus	90
Fowl plague	60–90
Lymphocytic chorio-meningitis	37–60
Rift Valley fever	30
Yellow fever	About 25
St. Louis encephalitis	,, 25
Louping-ill	15–20
Poliomyelitis	12–17
Other bacteriophages	10–35
Foot-and-mouth disease	8–12
Protein molecules (for comparison)—	
— egg albumin	9 by 3
— serum pseudo-globulin	About 7
— serum albumin	,, 5

FILTRATION OF THE VIRUSES

As the viruses are distinguished by their capacity to pass through filters which are impervious to ordinary bacteria it is essential to understand the types and limitations of the various filters used.

The term " filterable " was originally applied in respect to rather coarse earthenware filters of the Berkefeld type which would just hold back the smallest bacteria —e.g. *Bacillus prodigiosus*,[1] but must now be considered more in relation to the porosity of the filter used.

Four types of " filterable " organisms have generally been recognised :

(1) The viruses.

(2) Filterable bacteria—e.g. *Bacterium pneumosintes*, and similar anaerobic bacteria of the respiratory passages, which pass coarse filters; the pleuro-pneumonia organism ; and the *reported* filterable forms of the tubercle bacillus and other bacteria.

Saprophytic Filter-passing Organisms.—Laidlaw and Elford have described a group of filter-passing organisms isolated from London sewage. They are about 0·15μ in diameter and can be cultivated in broth with peptic digest of blood added. Three separate serological types have been noted. They resemble in some respects the pleuro-pneumonia organism.

(3) Certain spirochaetes and vibrios which, owing to their extremely slender form, their flexibility or motility, are able to pass through the coarser filters, especially if the time of filtration is prolonged.

It is important to note that the mere passage of an organism through an earthenware filter candle does not justify the term " filterable virus " being applied to it. When describing a virus or organism as filterable it is necessary therefore to indicate (1) the type of filter ; (2) the porosity of the filter ; (3) the pressure employed during filtration ; (4) the time of filtration ; (5) the pH of the fluid in which the material is suspended, and (6) the controlling organism with which the filter has been tested (*vide infra*).

Filtration is not the mere mechanical passage of particles of a certain size through slightly larger pores.

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[1] *B. prodigiosus* (*Serratia marcescens*) is a minute Gram-negative bacillus (*vide* Table *supra*) which, growing on culture medium, produces a red pigment.

but depends on physico-chemical factors. Thus the earthenware filter consists mainly of magnesium and calcium silicates, and according to the electric charge on the virus, so the particles will be adsorbed or be able to pass through. The more acid the solution the more liable are the particles to become adsorbed. In addition, the adsorption of a virus by protein or tissue, the amount of the latter substances present, the temperature and pressure of filtration, affect very profoundly the results obtained. Filtration, therefore, depends on many variable and uncontrollable factors, and inability to pass an earthenware filter is no measure of size.

It must also be emphasised that usually the presence of virus in the filtrate can be determined only by animal experiment or by cultivation in the embryo-chick (p. 606). As certain viruses are very susceptible to changes in hydrogen-ion concentration it is essential that the reaction of the virus material be carefully adjusted, otherwise the virus may be inactivated and so adjudged non-filterable.

TYPES OF FILTERS

(1) Earthenware (*a*) Coarse, *e.g.* Berkefeld.
  (*b*) Fine, *e.g.* certain Chamberland filters.
(2) Asbestos disks—Seitz.
(3) Collodion.

Berkefeld Filters

These are made from kieselguhr, a fossil diatomaceous earth found in deposits in Germany and other parts of the world. Filters made from this material are coarse —that is, have relatively large pores owing to the size of the granules forming the substance of the filter. They are made in three grades of porosity—namely V (viel) the coarsest, W (wenig) the finest and N (normal) intermediate. Of these, the Berkefeld V is the one usually employed, and it should not pass a small organism such as *B. prodigiosus.*

A similar type to the Berkefeld is the Mandler filter, manufactured in the United States.

(The coarser grades of the Chamberland filters, *vide infra*, are similar in porosity to the Berkefeld types.)

CHAMBERLAND FILTERS

These are made of unglazed porcelain and are produced in various grades of porosity. The finer grades will pass only certain ultramicroscopic viruses of extreme minuteness, such as the viruses of foot-and-mouth disease and of fowl plague. The most porous, L_1, allows many organisms to pass, being merely a clarifying filter. The next three, L_{1a}, L_2 and L_3, are comparable with the Berkefeld V, N and W candles respectively. The porcelain filters are used extensively for the removal of organisms from fluid cultures in order to obtain the bacterial toxin.

SEITZ FILTERS

This type consist of a disk of an asbestos composition (*vide* p. 88) through which the fluid is passed. Various sizes for laboratory work are available. After use the asbestos disk is discarded. The disks are supplied in three grades—termed clarifying, normal and "special EK." These filters are very convenient and useful when bacteria-free filtrates are required, and the larger sizes are especially valuable in the sterilisation of serum for making media. The normal and fine grades of disks do not allow the ordinary test bacteria—e.g. *B. prodigiosus*—to pass.

Similar disks are made in England, and are as reliable and efficient as the foreign ones. The grade GS corresponds to the EK, and the FCB to the K disks (*vide* p. 89).

COLLODION FILTERS

Elford[1] has devised a technique for preparing collodion membranes of graded porosity which he terms *gradocol* membranes, since they are products of graded coagulation of collodion. Collodion films show two types of structure :

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[1] For details of technique see Elford, W. J., *J. Path. Bact.*, 1931, **34**, 505, and *Proc. Roy. Soc.*, B., 1933, **112**, 384.

(a) *microgel*, which has a coarse structure visible microscopically, and

(b) *ultragel*, the structural elements of which are not resolvable by the microscope, but which is built up of particulate matter. Hitherto these membranes, although uniform, have not been sufficiently permeable for filtration work.

The *gradocol* membranes possess the permeability of the microgel type, but have the ultragel structure and are very uniform in their porosity. They are made from an acetone solution of collodion (nitro-cellulose) diluted with an ethyl-alcohol-ether mixture to which are added varying amounts of amyl alcohol. 75 c.c. of the mixture are poured into a shallow cell 20 cm. in diameter in a constant-temperature room (22·5° C.), allowed to evaporate for varying periods of one to three hours, and then washed over an extended period with distilled water. By varying the amount and composition of the collodion mixture, and the conditions of evaporation, permeable filters of average-porosity size (A.P.S.) ranging from 3μ down to 10 $m\mu$ or less have been prepared. It is possible to reproduce accurately at any time filters of any desired permeability and porosity. The technical details are elaborate and of extreme importance.

By means of these collodion membranes it has been possible to determine the size of many of the viruses.

Methods of filtration are dealt with on p. 645 *et seq.*.

When working with filterable viruses, controls must always be made of the filtered material. It must be cultivated both aerobically and anaerobically to show that no bacteria are present. Inoculation of animals susceptible to the virus should be carried out, and the filtrate should give rise to the train of symptoms characteristic of the disease.

HUMAN DISEASES IN WHICH THERE IS EVIDENCE THAT THE CAUSAL AGENT IS A FILTERABLE VIRUS

Epidemic poliomyelitis.
St. Louis encephalitis.
Australian encephalitis ("X disease"); probably due to same virus as that of louping-ill.
Certain other forms of encephalitis (*vide* p. 614).

Rabies (common also to dogs, cattle and certain other animals).
Lymphocytic chorio-meningitis.
Herpes febrilis (also neurotropic[1] in experimental animals).
Herpes zoster (also neurotropic).
Smallpox (variola), alastrim and vaccinia.
Chickenpox (varicella).
Molluscum contagiosum.
Common wart.
Foot-and-mouth disease (transmissible from cattle).
Trachoma.
Inclusion conjunctivitis.
Measles.
Influenza.
Common cold.
Yellow fever.
Rift Valley fever (common also to sheep and cattle —enzootic hepatitis).
Psittacosis (common also to parrots and certain other birds).
Phlebotomus fever.
Dengue.
Lymphogranuloma inguinale (or climatic bubo).
Mumps.

The following Human Diseases are considered to be due to Filterable Viruses, but the evidence is incomplete.

German measles (rubella).
Encephalitis lethargica.
Infective hepatitis.
Infective mononucleosis (glandular fever).
Primary atypical pneumonia.

Representative Diseases of Animals, Birds and Insects generally accepted as due to Filterable Viruses :

Foot-and-mouth disease of cattle.
Canine distemper.

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[1] A virus having an affinity for nervous tissues is referred to as *neurotropic* ; for the skin, *dermotropic*.

Feline infectious enteritis.
Sheep-pox and cow-pox (vaccinia—*vide supra*).
Swine fever (or hog-cholera).
Cattle plague (rinderpest).
Vesicular stomatitis of horses.
African horse sickness.
Borna disease of horses.
Equine encephalomyelitis (transmissible to man).
Infectious anaemia of horses.
Rabies (*vide supra*).
Pseudo-rabies or mad itch of cattle and other animals.
Louping-ill (encephalomyelitis) of sheep (transmissible to man).
Blue-tongue of sheep.
Nairobi sheep disease.
Contagious pustular dermatitis of sheep.
Encephalitis of silver foxes.
Swine influenza.
Infectious myxomatosis of rabbits.
Infectious fibromatosis of rabbits.
Salivary-gland disease of guinea-pigs.
Infectious ectromelia of mice.
Virus III of rabbits.
Filterable tumours of fowls—*e.g.* Rous sarcoma.
Fowl plague.
Newcastle disease of fowls.
Infectious laryngo-tracheitis of fowls.
Fowl-pox, including roup.
Pigeon-pox.
Canary-pox.
Leukaemia of fowls.
Psittacosis (*vide supra*).
Pacheco's parrot disease (*not* transmissible to man—*cf.* psittacosis).
Silkworm jaundice.
Sac brood of bees.

GENERAL CHARACTERS OF THE FILTERABLE VIRUSES

The filterable viruses differ broadly from the ordinary bacteria in many ways, and certain characteristic features are common to many of the series :—

1. Great infectiousness of the associated disease.
2. The production of a solid immunity, which may be of long duration.
3. Filterability.

4. Invisibility of some viruses by ordinary microscopic methods.

5. Non-cultivability *in vitro* by ordinary methods applicable to bacteria.

6. Resistance to glycerol.

7. The presence of "*inclusion bodies*" (or "*cell inclusions*") in lesions.

1. Great Infectiousness of the Associated Disease.—This is seen in smallpox epidemics, where, in spite of careful quarantine, cases continue to occur. Foot-and-mouth disease is another example; the difficulty of control is due to the amazing rapidity of spread, not only from animal to animal, but from one locality to another.

As a group, the viruses are markedly pathogenic, even in minute doses. The virus of yellow fever is capable of causing infection of the monkey in a quantity as small as 0·000001 c.c. of an emulsion of brain tissue from an infected animal. Incredibly small amounts of these viruses are capable of causing infection, as is shown by the rapid spread of the disease in spite of the utmost attempts at control.

2. The Production of Immunity.—In individuals surviving infection there is a high degree of immunity which often lasts for a long period of time. Advantage is taken of this in immunisation against smallpox and rabies by inoculation with attenuated viruses. In addition, specific antisera may be prepared, as in the case of hog-cholera and cattle plague, which are available for treatment. On the other hand, the immunity following infection may be of only short duration.

Prolonged Immunity		*Short Immunity*
Smallpox.	Measles.	Herpes febrilis.
Vaccinia.	Yellow fever.	Influenza.
Poliomyelitis.	Rift Valley fever.	Common cold.
Chickenpox.	Mumps.	Dengue.
		Trachoma.

It was at one time supposed that immunity could not be conferred by injecting dead virus into susceptible animals as is the case with killed-culture vaccines of bacteria, and it has been thought that living virus is absolutely essential for an immunity response. It has been shown, however, that formolised or carbolised virus can be used to produce active immunity against foot-and-mouth disease, louping-ill, rabies, canine distemper, rinderpest, etc..

3 and 4. FILTERABILITY and INVISIBILITY of these viruses have already been discussed.

5. CULTIVATION.—The filterable viruses are not cultivable on ordinary media, and their presence is recognised by their action on susceptible animals. It has been shown that a number of viruses can be grown in association with the proliferating cells of tissue cultures. Simplification of the process was achieved in the case of vaccinia virus by H. B. and M. C. Maitland, who obtained good growths, using fresh minced adult hen or rabbit kidney mixed with fresh serum and Tyrode's solution.[1] By such methods some viruses have been cultivated, but the conditions necessary for growth are not yet understood, and the procedure is somewhat empirical. It would appear that the obligatory intracellular parasitism of many of the viruses precludes their cultivation like the bacteria on inanimate substrates.

Several facts emerge from the mass of literature on the subject:

(1) Many of the filterable viruses can undoubtedly be cultivated *in vitro*.

(2) Different conditions of growth are necessary, and these must be provided just as for the bacteria, whose oxygen and temperature requirements, nutrient materials, growth factors, optimum H-ion concentration, etc., must be studied. Thus, in

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[1] *Tyrode's solution.*—NaCl, 8·0 gm., KCl, 0·2 gm., $CaCl_2$, 0·2 gm., $MgCl_2$, 0·2 gm., $NaHCO_3$, 1·0 gm., glucose, 1·0 gm., NaH_2PO_4, 0·05 gm., distilled water, 1000 c.c. The solution is adjusted to pH 7·4 and sterilised by filtration through a Seitz filter.

the case of the common cold virus, anaerobic conditions in living tissues appear to be necessary ; the viruses of herpes and vaccinia multiply in surviving or disintegrating tissue and active cell proliferation is not essential, while the fowl-pox, psittacosis and fowl-plague viruses require actively growing tissue cells. Certain viruses grow irrespective of the nature of the tissue cultures, but the virus of foot-and-mouth disease requires definite tissues from certain species—*e.g.* the pads, lips, tongue or hairy skin of the embryo guinea-pig.

Certain viruses—*e.g.* of vaccinia, fowl-pox, canary-pox, influenza, psittacosis, etc.—can be cultivated in the chorio-allantoic membrane of the developing chick. This provides a comparatively simple and convenient " medium." For a full description of the technique reference should be made to the Medical Research Council's Special Report, 1936, No. 220, by F. M. Burnet. The procedure may be briefly summarised as follows. A fertile egg which has been incubated for 10–12 days is used. It is then examined by trans-illumination. The developing embryo can be observed as an opaque area and the air sac can also be outlined. The surface of the egg is thoroughly washed and treated with antiseptic. The shell is carefully perforated with pointed scissors, and the virus material is injected under the shell membrane. If more than 0·2 c.c. has to be introduced the air sac is first punctured to allow more space for the inoculum. The openings are sealed with sterile tissue paper and paraffin-vaseline, and the egg is replaced in the incubator for five or six days.

The lesions produced are opacities and thickening of the membrane ; later plaques of greyish-yellow thickening are formed. Massive lesions and death of the embryo may result. Some viruses, however, may be propagated serially without producing any visible lesions.

6. Resistance to Glycerol. — Ordinary non-sporing bacteria are killed by 50 per cent. glycerol in a comparatively short time. Many of the filterable viruses, on the contrary, retain their viability much longer in this material than in any other fluid when kept at 4° C.. The preservation of the vaccinia virus used prophylactically against smallpox is accomplished by this means. Other viruses which keep for prolonged periods in glycerol at 4° C. are : poliomyelitis virus, the virus of febrile herpes (which may be kept virulent in glycerol for more than one year), and the

virus of rabies. On the other hand, some viruses—*e.g.* rinderpest virus and virus III of rabbits—will survive for less time than certain bacteria. It is essential that the glycerol should be pure and free from mineral acids, esters and sulphates.

(It may be noted here that the filterable viruses infecting man and animals are very readily destroyed by heat and antiseptics, but may survive outside the body for considerable periods of time, and can also resist natural desiccation.)

7. Inclusion Bodies.—When lesions occurring in filterable virus diseases are examined histologically there are frequently found within the cytoplasm or the cell nucleus peculiar structures which may be of two types—namely :

(1) " Elementary bodies "
(2) " Inclusion bodies."

" Elementary bodies " appear to represent the actual virus, and these are exemplified by the " Paschen bodies " which are seen in smallpox and vaccinia lesions in the exudate as well as in the cells, and which may be demonstrated in the vesicle fluid of early vaccinia in calves. When suitably stained they appear as small round coccus-like bodies, about $0 \cdot 2\mu$ in size. Similar bodies have been found in varicella and herpes zoster, while they are demonstrable in the lesions of psittacosis, molluscum contagiosum, ectromelia, fowl-pox, etc.. They can be demonstrated by Paschen's or Castaneda's stain (p. 226) and by prolonged staining in Giemsa's solution, when they appear red or purplish. By comparison with photographs taken with ultra-violet light (Barnard), the effect of staining is to make them appear larger. The work of Paschen and Ledingham on the elementary bodies of vaccinia, and of Bedson on the elementary bodies of psittacosis indicates that these represent actual virus bodies.

"Inclusion bodies" may be:

(*a*) Cytoplasmic inclusions—*e.g.* the Negri body found in the nerve cells of the central nervous system in rabies, the Guarnieri body of smallpox, the Henderson-Paterson body or "molluscum body" of molluscum contagiosum and the Bollinger body of fowl-pox. These inclusion bodies are acidophile in nature, and with Mann's methyl-blue-eosin (*vide* p. 225) or Giemsa's stain are coloured pink by the eosin. The Negri bodies are round or oval, and vary considerably in size (*vide* p. 611). Guarnieri bodies are found in the cytoplasm of epithelial cells of the skin lesions of smallpox and vaccinia. They stain with acid dyes and are often seen lying in clear spaces in the protoplasm. They may vary in size from 1μ to $15–20\mu$, and may be as large as the cell nucleus itself. Similarly, Bollinger bodies are pink-staining, cytoplasmic inclusion bodies, round or oval in shape, and varying considerably in size.

(*b*) Intranuclear inclusions: which can be demonstrated by Giemsa's stain, polychrome methylene blue and eosin, and Mann's stain, in the following diseases:—

Herpes febrilis	in the epidermis.
Chicken-pox	
Herpes zoster	
Pseudo-rabies	in the brain.
Borna disease	
Yellow fever.	in the liver.
Rift Valley fever	
Pacheco's parrot disease	

Virus III infection of rabbits—in the cornea, skin and testis.
Guinea-pig salivary gland disease—in the ducts.

These intranuclear inclusions are acidophile in nature and are stained reddish or pink, and vary considerably in size.

It has been shown that a single isolated Bollinger body (of fowl-pox) is infective for the fowl and therefore contains the virus. As, however, this inclusion body is comparatively large, and the virus of fowl-pox passes a filter, the Bollinger body does not indicate the actual form of the virus. When suitably stained and examined microscopically the Bollinger bodies are seen to consist of minute particles—termed Borrel bodies—in an amorphous matrix. These Borrel bodies are similar to the elementary bodies of variola and vaccinia, and are regarded as the virus. In fowl-pox, therefore, it would appear that the inclusion bodies consist of aggregates or colonies of the virus which is multiplying as an intracellular parasite.

By microdissection van Rooyen has been able to remove a molluscum body from the epithelial cell and on opening this inclusion it is found to contain a large number of elementary bodies surrounded by a gelatinous material.

The inclusion bodies were formerly supposed to consist of a mantle or sheath around the infective agents, and were known as "*chlamydozoa*," and while some inclusion bodies may represent degenerative or reactionary changes in the cell due to the action of the virus, recent evidence suggests that they are aggregates or collections of elementary bodies.

POLIOMYELITIS

Poliomyelitis is an acute infection, occurring chiefly in children, which affects the central nervous system with usually a special localisation in the anterior cornu of the spinal cord.

The disease can be communicated to susceptible animals by the inoculation of filtrates of infective tissue.

The virus passes through Berkefeld V and N filters. Its size is about 12–17 mμ. It is easily destroyed by heat (55° C. for 30 minutes), but can withstand the action of weak phenol for some days. In brain tissue, preserved in 50 per cent. glycerol at 4° C., it remains viable for years.

Growth of the virus occurs in tissue cultures of human-embryo tissue.

Intranuclear inclusion bodies—small eosinophile granules, 3–4μ in diameter—have been found in the nerve cells of fatal human cases.

Experimental Inoculation.—The disease can be transmitted to monkeys (*e.g. Macacus rhesus*) by the intracerebral or intravenous inoculation of human brain or spinal-cord material. (*Macacus cynomolgus* is specially suitable for dealing with a recently isolated strain.) The animals develop the typical disease, and show in the central nervous system histological lesions similar to those of human cases. The disease can also be transmitted from monkey to monkey.

Certain strains of the virus have been transmitted to the white mouse and Eastern cotton rat.

Infection has been thought to pass through the nose *via* the exposed ends of the olfactory nerves to the olfactory lobes of the brain. It has also been claimed that the virus may be carried in the nasopharynx of healthy persons who have been in contact with cases. Recent work has shown, however, that the virus is present in the excreta and that it can be recovered from sewage. It would appear that the infection may originate in the intestine and spread to the nervous system through the abdominal sympathetic nerves. It is doubtful if the occurrence of the virus in sewage is of epidemiological importance though the possibility of its spread by contaminated food or milk merits consideration. It has also been shown that the virus may be carried by flies.

The serum of convalescents contains neutralising antibodies, and convalescent serum is useful as a prophylactic, but its value in the treatment of the pre-paralytic or early paralytic stages of the disease is doubtful as once the virus has entered the cell, serum has no effect on it.

Attempts have been made to vaccinate against the disease with attenuated infective material, but this procedure is not without danger as such material may give rise to typical poliomyelitis in monkeys.

RABIES

This disease is communicated to the human subject by the bite of a rabid dog or other animal, the infective virus being present in the saliva of the animal. A paralytic form of rabies has been reported, *e.g.* in Trinidad, in which the infection is transmitted by the bites of vampire bats.

The size of the virus is 100–150 mμ and it is filterable through Berkefeld, Mandler and Chamberland L_1 and L_2 filters. Elementary bodies have not definitely been identified. Characteristic inclusion bodies—"Negri bodies"—occur in the cytoplasm of the nerve cells. They consist of round, oval or angular bodies varying in size from 0·5μ to 20μ, staining pink (with a slight purple tint) with Leishman's, Giemsa's, or Mann's stain. The larger Negri bodies may contain one or more dark-coloured granules. The nature of these structures is not yet elucidated, although their occurrence is a specific feature of rabies. The virus has been cultivated in tissue-cultures of chick-embryo brain. It is easily destroyed by heat (55° C. for 15 minutes) and chemicals, *e.g.* formalin, mercuric chloride, etc., but is resistant to glycerol. Phenol inactivates the virus and phenolised suspensions of infected rabbit's or sheep's brain are used for prophylactic immunisation.

Rabies can be transmitted experimentally to the dog, rabbit, guinea-pig, mouse and other animals. In rabbits, intracerebral inoculation produces paresis in seven to twenty-one days, and death occurs in fifteen to twenty-eight days. Infection can usually be produced through all the usual parenteral routes—intracerebral, intraneural, subcutaneous and intramuscular. *Post mortem* the characteristic Negri bodies are found in stained smears or sections of the brain, particularly in the hippocampus. The virus is localised in the nervous tissues and salivary glands and is transmitted through the body by the nerve fibres only. The incubation period in man is usually from thirty to sixty days. The virus becomes enhanced in virulence after repeated passage in the rabbit, until it kills regularly in six to seven days and then becomes the "fixed" virus (as designated by Pasteur).

When a person is bitten by a dog suffering from rabies, prophylactic treatment by the Pasteur method, or a modification, must be carried out without delay, and when there is any doubt as to the condition of the dog, an accurate diagnosis is essential. In this country where rabies is extremely rare, some veterinary surgeons recommend keeping the animal under observation for some time. If there is any suspicion of rabies, the animal should be killed, and if the laboratory is at some distance, the head is removed and forwarded in ice. In the laboratory the scalp is reflected, the skull is opened by means of sterile bone forceps and the brain removed with aseptic precautions. The hippocampus, which is situated in the floor of the lateral ventricle, is dissected out, smears are made by squeezing a portion of the tissue between two slides, and pieces are also fixed for histological examination. In addition, a suspension is prepared for animal inoculation. The smears are fixed in methyl alcohol for five minutes, and stained by Leishman's or Giemsa's method (*vide* pp. 217, 218) or by Mann's stain. For sections, the tissue is fixed in formalin (*vide* p. 227) and stained as above. The diagnosis depends on the finding of the characteristic Negri bodies in the cytoplasm of the nerve cells. Intracerebral inoculation of the rabbit with brain emulsion is carried out as described on page 238, and if the virus is present paresis and death occur as described above. The original Pasteur treatment of persons bitten by a rabid dog was to inject a suspension of spinal cord from a rabbit infected with "fixed" virus, the virulence of which had been reduced by drying. The first injection was a suspension of cord dried for twelve days, and subsequent daily injections of progressively more virulent nervous tissue were given. This live vaccine has been largely superseded by the phenolised virus described above.

Pseudo-rabies (Aujeszky's Disease—Mad Itch)

This is a disease affecting the central nervous system. It occurs in dogs, cattle, horses, pigs, sheep, rats and certain other animals, but is not transmissible to man. An important point of differentiation from rabies is that the animals suffer from intense itching of the hind-quarters. It also differs in that the animals are not aggressive. The virus is not strictly neurotropic for it may be demonstrated in the blood and organs. It is not found in the saliva. Antigenically it is distinct from the virus of rabies and has no connection whatsoever with this disease. It may be cultivated by tissue culture methods. The size of the virus is 100–150 mμ. There is evidence that the disease may spread from rats to pigs and from pigs to cattle.

HERPES FEBRILIS

If the clear fluid from a herpes vesicle be inoculated on to the scarified cornea of a rabbit, there follows a severe keratitis which can be propagated indefinitely through a series of animals. Some of the rabbits show cerebral symptoms and later die of encephalitis. Herpes virus is present in the brain uncontaminated by other organisms. It is very infective for rabbits, and recovered animals show a marked immunity.

The virus is filterable through Berkefeld and coarse Chamberland filters. Its size is about 125 (100–150) mμ. It can be cultivated easily in minced rabbit testis with rabbit plasma and Tyrode's solution, and in the chorio-allantoic membrane of the developing chick. Even after prolonged culture the virus retains its neurotropic properties. In the corneal and nerve cells of infected rabbits, nuclear inclusions ("Lipschütz bodies") may be demonstrated. These are acidophile intranuclear bodies, at first small, but later they coalesce and occupy the greater portion of the nucleus. They are similar to the inclusion bodies in other virus diseases. Typical inclusions can be demonstrated in the cells of the culture.

(*Herpes genitalis* is due to a virus similar to that of herpes febrilis.)

HERPES ZOSTER

This disease is characterised by an erythematous rash, later becoming vesicular, its distribution corresponding to that of a sensory nerve. The trunk is most frequently affected, on one side only, the distribution of the eruption being that of intercostal nerves (shingles). Other areas affected are the head and genito-urinary region. As compared with varicella to which it is related epidemiologically (*vide infra*), herpes zoster is more frequent in adults, and not so common in children.

The condition is aetiologically distinct from herpes febrilis and the causal agent is identical with or very closely allied to the virus of varicella. Elementary bodies of approximately $0{\cdot}25\mu$ in diameter can be demonstrated in the vesicle fluid. Intranuclear acidophile inclusions have been observed in epithelial cells. The virus can be cultivated in the chorio-allantoic membrane of the developing chick where characteristic " pocks " are formed.

In addition to its dermotropic properties the virus is neurotropic, as lesions are also found in the posterior root ganglia.

ENCEPHALITIS

The different types are: (1) Encephalitis lethargica, (2) St. Louis encephalitis, (3) Australian "X" disease, (4) Japanese encephalitis, (5) West Nile encephalitis. (6) Russian spring-summer encephalitis, (7) Equine encephalomyelitis, transmissible to man.

Encephalitis lethargica.—Little is known of the virus of this disease except that claims have been made of its occasional transmission to rabbits and monkeys. It is probable, however, that the virus of herpes febrilis was responsible for the infection in these animals. Much confusion has arisen in experimental work on this disease when rabbits have been used, because these animals suffer naturally from a spontaneous meningo-encephalitis, and this condition has been mistaken for the experimental disease. The precise aetiology of encephalitis lethargica is not yet known.

St. Louis Encephalitis.—This form of encephalitis was first defined by the isolation of the causative virus in an epidemic at St. Louis, U.S.A. The virus is transmissible to monkeys and mice by intracerebral inoculation. It is about 25mμ in size and passes Berkefeld V and N, and Seitz filters. It grows well in the chorio-allantoic membrane of the chick and in certain types of tissue culture. The disease, which is transmitted by mosquitoes, has no relationship to encephalitis lethargica. There is some evidence that wild and domestic birds (*e.g.* poultry) may constitute reservoirs of the infection.

Australian " X " Disease.—The virus of this condition has been shown to be closely related to, if not identical with, that of louping-ill (p. 638), while it also has some relationship to the virus of Japanese encephalitis, type B.

Japanese Encephalitis.—Two types of encephalitis have been described in Japan, the *lethargica* (type A), and " summer " encephalitis (type B), which is probably mosquito-borne. The virus of the latter condition is probably about 20–30μ in size, and passes Berkefeld W and Seitz E.K. filters. The virus grows in tissue culture and in the chorio-allantoic membrane of the developing chick. It is destroyed at 55° C. in 30 minutes but remains virulent in glycerol for many months. The condition can be transmitted to rabbits, mice, guinea-pigs and monkeys.

West Nile Encephalitis.—The virus has a slight antigenic relationship to that of Japanese encephalitis, type B.

Russian Spring-summer encephalitis is closely related to louping-ill (*vide supra*) and is transmitted by ticks, *e.g. Ixodes persulcatus.*

Equine encephalomyelitis.—See p. 640.

ACUTE LYMPHOCYTIC CHORIO-MENINGITIS

In this condition the patient becomes acutely ill with symptoms of meningitis, but recovers in a short time. Fatal cases are very rare.

The cerebro-spinal fluid shows an increase in lymphocytes, usually about 200 per cmm. but rising sometimes to 1500. The spinal fluid is sterile by ordinary methods of cultivation.

The disease is due to a virus, 37–60 $m\mu$ in size, which passes Berkefeld, Seitz, and Chamberland L_1, L_2 and L_3 filters. It is present in the blood in the early stage of the disease and later in the spinal fluid when meningitic signs appear. Inclusion bodies, acidophile and intranuclear, have been described in the brains of infected animals.

The virus can be cultivated in the chorio-allantoic membrane of the chick but no recognisable lesions develop. The virus is easily destroyed by heat (at 55° C. for 20 minutes), but resists 50 per cent. glycerol for many months.

The disease can be transmitted by intracerebral inoculation in guinea-pigs, mice and monkeys. If mice are inoculated subcutaneously there is no apparent infection, but intracerebral injection is fatal in 7–9 days. Guinea-pigs exhibit only a non-fatal febrile reaction following infection by any route. The pathological condition is an acute lepto-meningitis affecting particularly the basal meninges and producing an intense exudation of lymphocytes throughout the entire ventricular system. The infection occurs naturally in mice and the virus is excreted in the urine and nasal secretion of these animals. The source of infection in man is still obscure. Mice act as the reservoir and the infection may be conveyed by the excreta and by dust, although in America it has been suggested that the disease is insect-borne and nymphs of ticks have been shown experimentally to act as transmitters.

Neutralising antibodies may be found in the serum of recovered persons and also in contacts. Similar antibodies occur in infected animals. The serum may be tested by mixing it with the virus and, after incubation for 4 hours, injecting the mixture intracerebrally into mice. All the strains isolated are serologically identical.

In diagnosing the condition the cerebro-spinal fluid and blood should be examined to exclude cultivable organisms, and cell counts carried out. Mice (from virus-free stocks) are inoculated intracerebrally with 0·02 c.c. spinal fluid. If the virus is present the animal becomes ill in about 6 days with ruffled fur and convulsive movements, dying about the 7th–9th day after injection.

The serum of recovered cases gives a complement-fixation reaction with infected guinea-pig spleen as the antigen. Complement-fixing antibodies appear much earlier than neutralising antibodies which may not be demonstrable till several months after recovery and may sometimes remain completely absent.

A "*pseudo-lymphocytic chorio-meningitis*" has been described in which the virus is 150–225 $m\mu$ in size, in contrast to the smaller dimensions of the virus above described. The diseases also differ immunologically.

VARIOLA (SMALLPOX) AND VACCINIA (COW-POX)

Variola or Smallpox is due to a filterable virus, although filtration experiments have not always been successful owing to the adsorption of the virus by the filter candle and protein material in the suspension. In the skin lesions, inclusion bodies, "Guarnieri bodies," may be seen and also minute granules, "elementary bodies" or "Paschen bodies." Similar inclusion and elementary bodies are found in vaccinia (*vide infra*).

The virus from cases of smallpox does not reproduce the disease in the ordinary laboratory animals, but only a slight local lesion. In the monkey, however, material from a smallpox pustule, when inoculated intracutaneously, may produce a typical localised "pock," though not a general eruption.

A solid immunity is produced by an attack of the disease, or by vaccination with vaccinia virus.

The *vaccinia* or *cow-pox* virus (*vide* p. 32) is closely allied to, if not identical with, that of smallpox. The virus is not usually filterable. Its size is 225 mμ. Lesions are readily produced in calves and rabbits. "Vaccination" in human beings is the application of vaccinia virus to the scarified skin, with subsequent development of a pustule or "pock."

Various strains of vaccinia virus have been maintained over many years for smallpox immunisation and though some of these may originally have been derived from spontaneous cow-pox, others probably represent the variola virus modified by passage in calves.

Downie[1] has shown by cross-absorption of immune sera with elementary body suspensions that there is an antigenic difference between the viruses of *spontaneous* cow-pox and vaccinia. The results of agglutination, complement-fixation and neutralisation tests with absorbed sera suggest that although the antigens of cow-pox and vaccinia viruses are very much alike there are qualitative differences in the heat-labile component (*vide* p. 620). These, and differences in the lesions produced experimentally show that the virus of *spontaneous* cow-pox is not the same as the strains of vaccinia virus examined.

The inoculation of rabbits or calves with the virus after scarification of the skin is followed by a red swelling on the third day, which later becomes papular. By the fifth day the papules have developed into vesicles filled with clear fluid which soon becomes cloudy and purulent. About 8–10 days after inoculation scabbing occurs. Such reactions are obtained even with a high dilution of vesicular fluid—*e.g.* from 1:1000 up to even 1:100,000. If the virus be injected intravenously in large amounts into the rabbit, generalised lesions occur not only over the skin but in the internal organs. In the epithelial cells after cutaneous inoculation of the rabbit, large cytoplasmic inclusion bodies (Guarnieri bodies) may be seen, and in the early stages enormous numbers of elementary bodies (Paschen bodies) about 0·2μ in size (*vide supra*).

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[1] Downie, A. W., *Brit. J. Exp. Path.*, 1939, **20**, 158.

These elementary bodies were first noted and described by Buist of Edinburgh in 1886. The biological name *Buistia pascheni* has been proposed for them as they appear to be distinct organismal entities and represent infective units.

They have been found to consist of protein, carbohydrate and fat, with small amounts of copper, riboflavin and biotin. This is illustrative of the constitution of the larger viruses which approach in size to the bacteria. As observed by the electron microscope the virus bodies of vaccinia appear to be rectangular in shape as contrasted with the rounded bodies of varicella and herpes febrilis.

Elementary bodies have been separated and purified by high-speed centrifuging (Ledingham), and they can be agglutinated by a specific antiserum from recovered animals, while complement-fixation tests can be carried out with the separated bodies as antigen.

Cultivation.—The virus of vaccinia has been successfully cultivated, first in a tissue culture and later by a simplified method introduced by H. B. and M. C. Maitland. The medium consists of minced fresh adult kidney, either from a hen or a rabbit, Tyrode's solution and fresh serum. Bacteria-free vaccinia virus is mixed with 0·3 c.c. fresh minced kidney tissue, 3 c.c. fresh rabbit serum is added, and the whole diluted to 10 c.c. with Tyrode's solution. The mixture is distributed in 2 c.c. amounts in Carrel flasks and incubated at 37° C.. The kidney cells do not proliferate but remain alive for five to six days, and in association with them multiplication of the virus takes place. The virus likewise has been cultivated in a simple medium of chick embryo and Tyrode's solution. The virus also grows well in the chorio-allantoic membrane of the developing chick. Elementary bodies (Paschen bodies) have been demonstrated in cultures.

Vaccinia virus for vaccination is usually obtained by inoculating the scarified shaved skin of the calf with pustular material from the rabbit similarly inoculated with the virus. (The virus is maintained by alternate inoculation in calves and rabbits.) About the fifth day, when the lesions are pustular, the scarified area is scraped with a spoon, and mixed in a mortar with glycerol-saline. After storage in the cold for some time, the number of extraneous organisms rapidly diminishes, and the resultant product which contains the virus is called "calf-lymph."

Vaccination with the vaccinia virus in the human subject produces a localised pustule and confers immunity against smallpox.

Recently it has been advocated that instead of virus derived directly from animals, cultivated virus (from the developing chick or chick embryo medium) should be used for smallpox immunisation, the material being injected intracutaneously.

Serological Diagnosis.—Craigie and Tulloch's modification of Gordon's precipitation test is useful in distinguishing smallpox from chickenpox. The crusts from the suspected case are dried in a desiccator overnight and ground in an agate mortar. The powder is weighed, triturated with saline, allowed to stand for an hour and the mixture is centrifuged. Dilutions of the crusts, 1 in 100 to 1 in 800 are made, and to these are added various dilutions (1 in 20 to 1 in 80) of immune sera obtained by injecting rabbits with saline extracts of vaccinia crusts from a vaccinated rabbit. On incubation, if the case is one of smallpox, flocculation will occur in some of the tubes, there being optimum dilutions of crusts and serum which give the maximum flocculation. Chickenpox crusts yield no flocculation. A similar experiment with vaccinia crusts from the rabbit is set up at the same time to serve as a control.

Complement-fixation tests are also positive with smallpox or vaccinia crusts and a known immune rabbit serum. This test has been strongly recommended by Craigie and Wishart[1] as not only is it 8–10 times more sensitive than the flocculation test but is more easily carried out. The crusts of 6–8 lesions (0·008 grams) are sufficient, and are prepared as described for the flocculation test. The test is carried out like the Wassermann reaction. A disadvantage is that occasionally crusts may show marked anticomplementary properties.

It may be noted here that the antigen of the vaccinia virus has been shown to be composite, and two components heat-labile and heat-stable (at 70° C.) respectively have been differentiated. Corresponding antibodies have been demonstrated in immune sera.

Microscopic Diagnosis.—Recently van Rooyen and Illingworth (*Brit. Med. Journ.*, 1944, ii, 526) have shown how early smallpox can be diagnosed and differentiated from chickenpox by the recognition of the characteristic elementary bodies in films from the skin lesions, Paschen's staining method being used (p. 225).

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[1] For full details, see Craigie, J., and Wishart, F. O., *Canad. Publ. Hlth. J.*, 1936, **27**, 371.

Post-Vaccinal Encephalitis

Within a fortnight of vaccination an acute disseminated encephalomyelitis may supervene in a very small percentage of cases. A disease identical in its clinical and histological characters has been recorded following other infective diseases, such as smallpox and measles, and occasionally in non-exanthematous cases.

Clinically there is paralysis, at first flaccid and later spastic, while meningeal symptoms are frequently noted in children. Histologically the outstanding characteristic is the demyelination of the areas round the blood-vessels. This is noted particularly in the white matter of the cerebrum, mid-brain, and pons, and also in the cord. Congestion and infiltration with mononuclear cells are also present.

It was originally thought that the encephalitis was actually due to the vaccinia virus affecting the central nervous tissue, but it is now considered that post-vaccinal encephalitis is an entirely different disease, due to a separate virus or toxin, but induced by the antecedent disease. It has been suggested that the condition is due to allergy with resultant vascular thromboses which produce the characteristic areas of demyelination round the blood vessels.

VARICELLA

Varicella (chickenpox) shows close epidemiological association with herpes zoster and like the latter is due to a filterable virus. Thus, cases of herpes have been followed by outbreaks of chickenpox. In the lesions of varicella minute bodies similar to Paschen bodies can be demonstrated, and intranuclear inclusions like those in herpes zoster have been observed in the epidermal cells. Serological experiments have shown a pronounced cross-immunity between the varicella and herpes zoster viruses, which are now regarded as being identical, or very closely allied.

Thus, material from zoster cases may produce typical varicella when inoculated into susceptible persons. This is not the case in children who have had varicella, while children inoculated with zoster material are subsequently immune to inoculation with varicella.

MEASLES

Little is known about the causal agent of measles. The disease is not communicable to ordinary laboratory animals, but it has been claimed that under favourable circumstances a modified form of measles may be produced in the monkey by inoculation with blood taken at the height of the disease. The experimental condition has been transmitted through a series of animals and the virus shown to be filterable.

It has been claimed that the virus can be established in rabbits by testicular inoculation and after passage in these animals on transfer to the human subject can reproduce the typical disease.

The virus has also been isolated by the intratesticular inoculation of guinea-pigs with human blood, and can be maintained by serial intratesticular passage.

The virus can be grown in the chorio-allantoic membrane of the developing chick, infective blood or throat filtrates being used as the inoculum. The cultivation can be carried out serially and the disease is reproduced in young monkeys from the culture.

Such cultivated virus after numerous repeated passages in eggs becomes attenuated and has been used by some investigators as a vaccine for active immunisation of children.

Serum from convalescent patients has marked protective powers, and if injected in amounts of 5–10 c.c. before the fifth day after exposure to infection will effectively prevent the disease in young children. If given after this time, the disease is modified and the risk of complications considerably reduced.

If convalescent serum is not available, serum from young adults (" Adult measles serum ") who have had measles in childhood is also efficacious in attenuating an attack of the disease, although a much larger dose must be given.

The doses of "Adult measles serum" are :—Under 3 years of age, 10 c.c. ; 4 years of age, 15 c.c. ; 5 years of age, 20 c.c. It is not recommended that "Adult measles serum" should be given to children over 5 years of age owing to the large quantities of serum required. If prophylaxis or attenuation of the disease is desired in such children, convalescent measles serum, 15–20 c.c., should be used. A preparation made from human placenta termed "Immune globulin" is also used in measles prophylaxis. The dose is 2 c.c. given intramuscularly.

COMMON COLD

The causative agent of the common cold is a filterable virus, and can be transmitted to man and to chimpanzees. Other animals are not susceptible. By passage through persons its virulence is raised so that colds of unusual severity can regularly be produced. The virus has been cultivated in a medium of minced ten-days-old chick embryo and Tyrode's solution. Cultures were obtained by concentrating nasal washings from a case of enhanced virulence by vacuum distillation ; this inoculum was added to the medium, covered by a vaseline seal and incubated at 37° C. for four to nine days. A series of subcultures was made, and infection of human volunteers was obtained with material from the twenty-seventh subculture.

INFLUENZA

Wilson Smith, Andrewes and Laidlaw have shown that influenza is due to a filterable virus and that ferrets are susceptible to the disease. Filtrates from nasal washings of influenza patients, when instilled intranasally into ferrets, produce a characteristic train of symptoms. After forty-eight hours the temperature rises, the animal becomes "out-of-sorts," and on the third day catarrhal symptoms appear. The eyes are watery and there is a variable amount of watery discharge from the nose. The disease lasts for a few days only and is not fatal. It can be transmitted serially in ferrets either by contact or by intranasal instillation of virus material. No other method of inoculation induces the disease. Immunity in the ferret persists for about three months. The virus is found only in the nasal mucous membrane of the animal and not in blood, lymph glands or spleen. The infection can be transmitted to mice from ferrets. In mice lung lesions occur, varying from small haemorrhages to complete consolidation. The infection has also been transmitted experimentally to hedgehogs. By filtration through gradocol membranes it has been estimated that the size of the virus is about 80–120 mμ.

A few strains of the influenza virus have been adapted for growth in the chorio-allantoic membrane of the developing chick. At first the lesions associated with growth of the virus are trivial but the virulence increases by passage. The virus also multiplies rapidly in the allantoic fluid and this is now the main source of virus used in experimental work.

The disease can be induced in ferrets only with material from acute influenza cases, and not from normal persons or those with common colds. Serum from recovered animals neutralises the virus, while human sera, particularly from influenza convalescents, neutralise the ferret and mouse virus.

Vaccination of the human subject against influenza with formolised infected tissue (mouse lung) has been employed. Virus grown in the allantoic sac has also been used. Although this method has given satisfactory results in mice its efficacy in man has not been proved. It has further been recommended that attenuated virus from allantoic culture should be instilled intranasally to " block " the path of infection. The immunity to influenza virus, either natural or acquired by vaccines, is of short duration only.

By immunisation of horses an antiserum has been produced which in mouse experiments is capable of neutralising or inactivating the virus. Recent experiments, both in this country and in the U.S.A., have indicated that there may be several types of the virus differing in antigenic characters.

Although the influenza virus has been isolated from many outbreaks of the disease, there have been large groups of cases in which the virus could not be demonstrated by inoculation of ferrets. Recently it has been agreed to term the original virus described above as " influenza A virus," and other types subsequently isolated as "B," "C," etc.. An influenza B virus has been isolated in New York; it is completely distinct serologically from the A virus, and is much more difficult to transmit to ferrets and mice. Sera from patients convalescent from B virus influenza show neutralising bodies to this virus. From serological evidence it has been thought that several outbreaks of influenza in America have been due to the B virus. It should be noted that Hungarian strains are different from both A and B.

Recent work[1] has shown that there is a *virus-inactivating agent* present *in human nasal secretion* which is capable of neutralising all types of influenza virus, and also other viruses such as those of herpes, louping-ill, etc.. It has no action on the viruses of vaccinia, ectromelia, fowl-pox, psittacosis, or pseudo-rabies. There is a slight but significant action on the virus of poliomyelitis. Inactivation proceeds at 37° C. but not at 0° C., while activity is destroyed by boiling for 10 minutes. There is no correlation with the lytic agent, lysozyme, found in tears, etc..

In 1941 Hirst described the phenomenon of *agglutination of red cells of the fowl by* preparations of *influenza virus.* He infected chick embryos with the influenza virus and showed that the egg fluid contained an agglutinating substance. He further showed[2] that serum containing neutralising antibodies for the influenza virus (as determined by the mouse-protection test) inhibited the agglutination of fowl red cells and that such inhibition was specific for the particular type of virus concerned, either A or B. Normal sera also inhibit the agglutination reaction but in a much lower dilution. The agglutination test will demonstrate *in vitro* the rise of influenza virus antibodies in human serum after an attack of influenza, as readily as the mouse test, for which it is a simple substitute.

Influenza virus is very closely related to that of *swine influenza*, and although they are different antigenically there is a common antigenic factor. It should be noted that in swine influenza two separate agents can be found—namely, the virus, and *B. influenzae* (*suis*). The virus alone produces a mild almost unrecognisable disease, while the bacillus by itself is harmless. In natural swine influenza the two agents act together, the virus being the essential factor. When the virus of swine influenza is inoculated in the ferret, however, the disease is not modified by simultaneous inoculation of *B. influenzae* (*suis*).

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[1] Burnet, F. M., Lush, D., and Jackson, A. V., *Brit. J. Exp. Path.*, 1939, **20**, 377.

[2] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

Equine influenza resembles in some respects human influenza. The virus is present in the blood and secretions. Carriers probably occur.

YELLOW FEVER

The infection is spread naturally by the bite of certain mosquitoes, e.g. *Aëdes aegypti*, *Aëdes simpsoni* (in Uganda), and *Aëdes leucocelaenus* and *Haemagogus capricorni* (vectors of "Jungle Yellow Fever" in Brazil). The disease can be communicated to *rhesus* monkeys either through the bites of infective mosquitoes or by injecting emulsions of internal organs—*e.g.* the liver—from fatal cases. The virus will pass fine filters, its size being 18–27 mμ. It is present in the blood and organs, particularly in the liver, in which organ well-defined intranuclear inclusion bodies ("Torres bodies") are to be found. The serum of convalescent patients has strong protective properties.

The virus can be transmitted to the brains of mice where it produces an encephalitis, and after twenty or more passages becomes "fixed" (with neurotropic properties). This virus will produce encephalitis in monkeys and some rodents, such as guinea-pigs, field voles, squirrels, etc.. The neurotropic virus leads to the production of immune bodies which protect against the viscerotropic strain.

The virus has been cultivated in the chorio-allantoic membrane of the developing chick, in a chick embryo medium, and in the carcinoma cells of tumour-bearing mice.

A considerable amount of work has been carried out on prophylactic immunisation against yellow fever. Several forms of vaccine have been adopted ; but that in present use is the "aqueous base" living vaccine consisting of an aqueous extract of 10–11 days chick embryos infected with the attenuated strain of the virus ("17 D"). This preparation has a much higher virus content (at least 66,000 M.L.D. per c.c. for the mouse) than previous vaccines, and contains no human serum (*vide* p. 634). The dose is 0·5 c.c. Immunity is produced after 15 days and full protection is maintained for at least 4 years.

In surveys of yellow fever districts, tests of immunity to the disease are made by the "*mouse protection test.*" The mouse is injected intraperitoneally with the serum to be tested and "fixed" virus (infected mouse brain), while a small quantity of starch solution is injected intracerebrally. If the serum contains no antibodies the mouse dies of encephalitis within ten days.

Psittacosis

This is an epizootic disease of parrots and parakeets which may affect human beings, often with fatal results. Recent work suggests that other species of birds may suffer from the disease under natural conditions ; its occurrence in fulmar petrels has been established and shown to be the source of human cases in the Faroe Islands and Iceland. The disease can be transmitted experimentally to birds of the parrot family and many species of finch, and to mice, monkeys and guinea-pigs. The virus is present in the organs of the infected host and is found in greatest quantity in the spleen and liver, and also in the lungs when these organs are involved, as is often the case in human psittacosis. It is between 220 mμ and 330 mμ in size and passes through the coarser filters such as the Berkefeld V and Chamberland L_{1a}. Its resistance to glycerol, though greater than that of the non-sporing bacteria, is by no means so great as that of most viruses. Psittacosis virus is present in the tissues in the form of visible bodies which stain readily with Giemsa's solution and by rickettsial staining methods such as Castaneda's. In smear preparations of virulent material stained by Castaneda's method, round or slightly oval elementary bodies are seen (0·2–0·8μ in size) the clear blue colour of which contrasts with the pink of the cells and background ; these bodies are found lying free or inside reticulo-endothelial cells which are sometimes filled to bursting point. The elementary bodies can be freed from cellular material by submitting a suspension of virulent material to fractional centrifugation. The washed particles so obtained are agglutinated specifically by an anti-psittacosis serum and fix complement with it, thus showing that they are, in fact, the virus. Like all other viruses psittacosis virus requires living cells for its multiplication and it appears to grow within the cells. Bedson and Bland have shown that this virus, when multiplying, passes through a regular sequence of morphological changes. The elementary body or filterable phase on entering a cell changes into a larger form 0·8μ to 1μ in size, which appears to multiply by division. As multiplication proceeds there is a progressive diminution in size of the virus bodies and in 48 to 72 hours after infection of the cell, they are all once more of the minute elementary type. The multiplying virus also forms a more or less compact colony in the cytoplasm of the cell consisting of virus bodies imbedded in a homogeneous matrix of basophile material. (A similar developmental cycle has been demonstrated in the case of the viruses of inclusion conjunctivitis and lymphogranuloma inguinale.)

Complement-fixation tests with an antigen of infected mouse spleen emulsion, or cultures of the virus in Rivers' tissue culture medium or in the chorio-allantoic membrane of the developing chick are employed for the diagnosis of psittacosis in the human subject and for detecting the disease in imported parrots. The mouse spleen antigen described below is the most convenient to use. Preliminary observations suggest that human convalescent serum, and a highly potent antiserum from the goat may be of value in the treatment of human psittacosis.

Diagnosis of Psittacosis

(1) *Suspected human case of the disease.*—(*a*) *Acute.*—Emulsify sputum (which contains the virus) in saline or phosphate buffer solution (pH 7·4), and centrifuge to deposit cells and the majority of the bacteria. Inoculate at least 2, and preferably 4 to 6 mice intraperitoneally each with 0·5 c.c. of the supernatant fluid. If the case is one of psittacosis, some or all of the mice will have died in 7–10 days showing signs of experimental psittacosis, namely a glairy peritoneal exudate, and enlargement of the spleen and liver. Smears from the exudate and spleen, when stained with Castaneda's or Giemsa's stain, show typical virus elementary bodies. It is recommended for confirmation also to inoculate an emulsion of the infected mouse spleen into two further mice which should die in 7–10 days with similar symptoms, the virus bodies being demonstrated microscopically in the peritoneal exudate and spleen.

If the mice inoculated with the human material have not died within 10 days, they are killed, the spleen is examined microscopically for virus bodies and an emulsion of the spleen is inoculated intraperitoneally into 4 to 6 mice. If none of these die within 10 days, the case is presumed negative. If any of the mice show signs of disease or die they are examined as above, and further mice inoculated with spleen emulsion to confirm the diagnosis.

(*b*) If the case is *past the acute stage* or is *convalescent*, a complement-fixation test should be carried out. This has been shown by Bedson to be very reliable and specific. The essential factor for success is an antigen rich in virus. The antigen is derived from mouse spleens infected with a virus which is highly pathogenic for the mouse. The spleens from the infected animals are first examined microscopically with Castaneda's or Giemsa's stain to determine the presence of abundant virus elementary bodies. A 5 per cent. emulsion of the spleen is made in buffered saline and allowed to sediment overnight. The supernatant fluid is removed and centrifuged at high speed to throw down the virus. This is resuspended in buffered saline to the same volume and placed in the steamer for 20 minutes. This boiled fluid or " coctoantigen " is used for the complement-fixation test. The test is carried out in the usual way, controls with normal and known positive psittacosis sera being included. Antigen controls are also included, spleens of normal mice (or of mice dead of another virus disease such as ectromelia) being used and treated in the same way.

(2) *Examination of birds.*—If a dead bird is received the spleen is examined with the naked eye for enlargement. In a budgerigar the spleen may be so small that only a microscopical examination can be made and the liver is then used for inoculating mice. In the case of a parrot the spleen is large enough for both smear preparations and animal inoculation. A suspension of the spleen or liver is inoculated into 4 to 6 mice which are observed for 10 days. Animals dying within that period, if positive, show a glairy peritoneal exudate and enlarged spleen, in which the virus can be seen microscopically after staining by Castaneda's or Giemsa's methods. Further animals are inoculated with spleen emulsion and should die within 7–10 days with macro- and micro-scopic evidence of psittacosis. If the mice inoculated from the bird have not died within 10 days they are killed, the spleens are examined microscopically and spleen emulsion is injected into further mice. If these are not dead within 10 days, the case may be regarded as negative. If the parrots have recovered, blood may be obtained from the superficial ulnar vein under ether anaesthesia, and a complement-fixation test carried out as described above.

The relationship of psittacosis virus to other viruses of similar morphology.—Recent work has shown that in addition to birds of the parrot family and fulmar petrels, pigeons and the domestic fowl may suffer from psittacosis. Since the strains of virus from these different sources, though similar in their antigenic structure, differ in their host-range and pathogenicity, it would be more correct to speak of viruses of the " psittacosis group " rather than of psittacosis virus. It has been recognised for some time that there is a close resemblance in morphology and staining characters of the viruses of psittacosis, lymphogranuloma inguinale (p. 631), trachoma (p. 632) and inclusion conjunctivitis (p. 633). They all stain by Castaneda's method and might be termed the " Castaneda-positive group." Included now in this group are the mouse pneumonia virus of

Nigg[1] which occurs as a latent infection in many mouse stocks and can be activated by a variety of means, and the viruses of meningo-pneumonitis of Francis and Magill,[2] and of atypical pneumonia (p. 636) of Eaton, Beck and Pearson.[3] The virus of meningo-pneumonitis came from a ferret which had been inoculated with nasopharyngeal washings from a case of influenza; it behaves like a strain of psittacosis virus of pigeon origin. The exact relationship of the virus of Eaton and his associates to the strains of psittacosis virus of parrot and pigeon origin is undecided.[4] It appears that this resemblance is more than superficial since recent American work has shown that all these viruses are antigenically related. This has an important practical bearing on the serological diagnosis of infections caused by them. For example, the complement-fixation test for psittacosis or lymphogranuloma inguinale can no longer be considered specific; the sera from cases of trachoma and inclusion conjunctivitis may fix complement with a psittacosis antigen, and the Frei test (p. 631) may be positive not only in lymphogranuloma inguinale but also in infections due to the psittacosis group. More than ever the interpretation of these laboratory investigations has to be made in the light of the clinical and epidemiological findings.

Pacheco's disease of parrots though producing a similar clinical picture in the bird differs from psittacosis in many respects. It is not transmissible to other species of birds and does not infect man. The virus has not been seen but produces nuclear inclusions in affected cells.

Mumps

The infection is transmitted by the saliva. Injection of the saliva from early cases of mumps into Stenson's duct in monkeys produces, after an incubation period of six to eight days, a rise in temperature, swelling and oedema of the parotid gland and a lymphopenia. The disease can be transmitted in series by means of infective gland. The infection in the monkey has been transmitted to human volunteers. The virus is filterable through Berkefeld filters, resists desiccation and is preserved by 50 per cent. glycerol. It is neutralised by serum from recovered cases of mumps. No definite elementary bodies have been described. Estimation of the size of the virus has not been made.

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[1] *Science*, 1942, **95**, 49.

[2] *J. Exp. Med.*, 1938, **68**, 147.

[3] *J. Exp. Med.*, 1941, **73**, 641.

[4] For a discussion of the interrelationship of these viruses, see Beck, Eaton and O'Donnell, *J. Exp. Med.*, 1944, **79**, 66.

Lymphogranuloma Inguinale (or Climatic Bubo)

This disease is transmitted by venereal contact. It has been increasing in frequency both in this country and in the Continent. The appearance for a few days of a small primary sore on the external genitals is followed in males by a swelling of the lymph nodes in the groin ; in females where the primary sore is often situated in the vagina, the inflammatory reaction may affect the peri-anal tissues, leading to stricture of the rectum and elephantiasis of the genitals. The virus inoculated into the groins of guinea-pigs, monkeys and dogs produces a bubo ; inoculated intracerebrally into monkeys, mice, guinea-pigs, dogs and cats, it gives rise to meningo-encephalitis. Intra- and extra-cellular elementary bodies have been described in human and animal lesions, and also larger inclusion bodies. A life cycle similar to that of the virus of psittacosis has been described. Apparently the elementary bodies after invading cells develop into largerforms which by successive divisions constitute compact masses and these finally divide into large numbers of elementary bodies. The size of the virus is estimated to be 125–175 mμ. It passes Berkefeld, Seitz and coarse Chamberland filters. The virus has been grown in tissue culture containing embryonic guinea-pig brain tissue and in the yolk-sac of the chick embryo. Serum from recovered cases is capable of neutralising the virus. Pus from infected lymph nodes, diluted 1 in 5 with saline and heated to 60° C. when inoculated intradermally in persons who have suffered from climatic bubo leads to a specific inflammatory reaction in the skin (Frei's reaction). Brain tissue from infected mice has been used for preparing the Frei reagent, but, if used, a control test with normal mouse brain should also be carried out. The yolk-sac culture described above is a very satisfactory antigen for the Frei reaction and complement-fixation tests.

Phlebotomus Fever

This disease of tropical and subtropical climates is transmitted by the sand-fly *Phlebotomus papatasii*. From experiments on the human subject the transmission of the disease by this insect has been established and evidence has been obtained that the virus (present in the blood) is filterable. The virus measures 160 mμ in diameter. It has been cultivated in the chorio-allantoic membrane of the developing chick.

Dengue

This infection is transmitted by the mosquito, *Aëdes aegypti*. Experiments have been carried out in the human subject in whom the disease has been reproduced by the injection of unfiltered and filtered blood. The virus apparently persists in the circulation for five days. It can pass fine Chamberland filters. The infection has also been transmitted to guinea-pigs. Immunity following an attack of dengue usually lasts for 1–4 years.

Molluscum Contagiosum

In the epithelial cells of the skin lesions large inclusion bodies, mainly acidophile in their staining reactions, can be observed. These have been called "molluscum bodies" or "Henderson-Paterson inclusion bodies." Material from the lesions stained by Giemsa's or Paschen's stain shows elementary bodies about 250 mμ in size.

It has been shown by van Rooyen that the molluscum body is a pear-shaped structure, about $20\mu \times 30\mu$ in size, inside the epithelial cell. By microdissection he has been able to isolate the molluscum body which on being opened is found to contain numerous elementary bodies suspended in a gelatinous fluid. The molluscum body apparently constitutes a phase in the life cycle of the virus. With filtrates from the lesions positive inoculation results have been obtained in the human subject.

Common Wart

This has been reproduced in the human subject by inoculation of the skin with Berkefeld filtrates from the lesions. The incubation period is long (1–20 months). Inclusion bodies have been described in the cytoplasm and nucleus of the cells.

Trachoma

Trachoma has been shown to be due to a virus which can be transmitted experimentally from one person to another. Filtration experiments with Berkefeld candles have been mainly negative, but positive results have been obtained with collodion membranes. The size of the virus is about 200 mμ. Characteristic inclusions — Halberstaedter - Prowazek bodies — are present in the conjunctival epithelium. Stained by Giemsa's method they may be blue, violet, or red in colour and are seen to contain small elementary bodies. Films from the conjunctival secretion stained by Giemsa's method show small reddish elementary bodies surrounded by non-staining material. The virus has not been cultivated. It is easily killed by heating at

55° C. for 15 minutes and dies rapidly outside the body. The disease can be transmitted to man but it is doubtful if animals have been successfully infected. Immunity to trachoma is of short duration. A small motile Gram-negative bacillus, *Bacterium* (or *Noguchia*) *granulosis*, has also been found associated with trachoma.

Inclusion Conjunctivitis

This term includes three forms of follicular inflammation of the conjunctiva, (*a*) swimming bath conjunctivitis, (*b*) conjunctivitis of the same type but independent of infection in swimming baths, and (*c*) non-bacterial ophthalmia neonatorum. Each of these is caused by the same virus and characterised by inclusion bodies in the cytoplasm of the epithelial cells of the conjunctiva. Swimming bath conjunctivitis is rarely seen in Britain but is common in the continent of Europe and in the United States. It is spread by persons with infection of the urethra (" inclusion urethritis ") and cervix uteri (" inclusion cervicitis ") urinating in the bath. The disease may also be acquired by contact with infected persons or infants suffering from inclusion conjunctivitis. Non-bacterial ophthalmia neonatorum is contracted during passage by the infant through the birth canal of the mother. (It may co-exist with a gonococcal infection of the conjunctiva). Thus, the virus of inclusion conjunctivitis localises primarily in the genito-urinary tract, and conjunctivitis is a secondary disease. From the urethral and cervical secretion inclusion conjunctivitis has been produced in the eyes of baboons. Infection can be conveyed to man and animals by bacteria-free filtrates. The virus resembles but is apparently distinct from that of trachoma.

The incubation period is about 8 days (3–12 days); first one eye is affected and then the other. There is a swelling of the lids, and the inflammation is at its height by the 4th or 5th day with a non-painful copious purulent discharge. The diagnosis is based on the finding of inclusions within the epithelial cells of the conjunctiva. The lids are everted and the conjunctival surface swabbed free from pus. A drop of adrenalin solution is applied, the conjunctiva is scraped with a scalpel without drawing blood, and films are made on slides. These are fixed in methyl alcohol and stained with dilute Giemsa's solution (p. 220) (1 drop of stain in 1 c.c. distilled water) for 24 hours.

The cellular inclusions show two phases (1) a minute " elementary " body, also found extracellularly, about 250 mμ in size and (2) an " initial " body, 300–800 mμ in size. The elementary body penetrates the epithelial cell and develops into the larger "initial" body which later divides up into elementary bodies and these infect new cells. The cycle of growth takes about 48 hours, and is similar to that of the psittacosis virus (p. 627). The elementary body is filterable through an Elford collodion membrane of average pore diameter of 450–650 mμ.

The virus does not resist drying and is quickly killed by heat at 58° C.. It has not been cultivated outside the body. Experimental infection can be produced in several species of monkey, including *Macacus rhesus* and the baboon, which develop a disease comparable with a mild human infection. The typical inclusion bodies are present in the animal lesions. In monkeys the virus dies out after several passages. Intracerebral inoculation in the mouse and rabbit fails to produce encephalitis, while the chorio-allantoic membrane of the chick cannot be infected. No general immunity is conferred by an attack. Neither viricidal antibodies nor agglutinins have been demonstrated in the blood of convalescents. Complement-fixation tests with psittacosis antigen and convalescent serum have been negative.

Infective Hepatitis

This disease which has an incubation period of approximately 20–40 days is presumed to be caused by a virus though the aetiological agent has not been definitely isolated. Transmission experiments in man suggest that a virus is present in the blood, duodenal-washings and urine up to about 48 hours after the appearance of jaundice. Recent reports from Germany state that the infective agent, which is very thermolabile, can be isolated from duodenal juice and urine in the pre-icteric phase by inoculation into the chorio-allantoic membrane of developing chicks, or into canaries. These results await confirmation.

From biopsy material obtained by liver-aspiration it has been possible to study the pathological process and it has been shown that in most cases the jaundice is due to a diffuse hepatitis and not to duodenal catarrh and obstruction of the common bile duct by mucus. The inflammatory lesions may be diffuse, zonal or mixed. The diffuse hepatitis usually heals completely and rapidly. When the disease runs a longer course some residual fibrosis in the portal zones may still be present after apparent recovery.

In recent years a clinically similar condition has occurred 60–130 days after the injection of certain batches of measles convalescent serum, yellow fever vaccine (containing human plasma), pooled adult serum, and mumps convalescent plasma. It has not been possible to reproduce the disease in any laboratory animal by the inoculation of these icterogenic blood products. The causative agent can pass through a Seitz filter and survive heating in a water bath at 56° C. for an hour. It is inactivated by $2\frac{1}{2}$ hours exposure to ultra-violet radiation consisting of 2650A° for 1 hour and 2537A° for $1\frac{1}{2}$ hours. It has been suggested that the agent is the same as that causing infective hepatitis and has been present in the blood of one of the donors. However in no instance has it been possible to trace such a donor in any incident reported so far and it is possible that an entirely different agent is responsible.

The same pathological picture is seen in both the above types of hepatitis.

Infective Mononucleosis (Glandular Fever)

The evidence, though incomplete, indicates that the disease is due to a virus. Thus, it has been shown that blood from cases injected into monkeys may produce a definite mononucleosis after an incubation period of 2 to 3 weeks, and in some experiments the infection has apparently been transmitted serially in monkeys while the virus has also been found to be filterable through a Seitz disk.

A laboratory method of diagnosis is the Paul-Bunnell test in which the serum of an infected or recovered person agglutinates sheep red cells. The test is performed as follows:—Heat the serum at 55° C. for 20 minutes. Make a series of doubling dilutions of the serum with saline in 0·5 c.c. amounts in 3 in. × $\frac{1}{2}$ in. tubes, ranging from 1 in 16 to 1 in 1024, as described on p. 251 for agglutination tests. A control tube containing only saline is included. Add to each tube 0·5 c.c. of a 1 per cent. suspension of sheep red corpuscles in saline, washed as for the Wassermann test. Shake the tubes thoroughly and incubate at 37° C. for 4 hours. Note which tubes show agglutination of the red cells and state the titre of the reaction in terms of the final dilution of the serum: 1st tube 1 in 32, 2nd 1 in 64, etc.. Normal serum may agglutinate in low dilutions. A suggestive titre is 1 in 128. A significant titre is 1 in 256. It should be noted that the reaction is negative in tuberculosis, leukaemia and Hodgkin's disease, but may be positive in cases of serum sickness. See **Appendix** for further references to the Paul-Bunnell reaction.

Primary Atypical Pneumonia

This condition, also referred to as "acute pneumonitis," "acute interstitial pneumonitis," or "virus pneumonia," is caused by a variety of viruses, some of which have been identified. Of these the viruses of the psittacosis group (*vide* p. 629) have first claim for consideration, since their aetiological connection with atypical pneumonia is the most firmly established. The available evidence, which is mainly serological, suggests that they are responsible for about 5–15 per cent. of cases. There is also some evidence that the virus of lymphocytic chorio-meningitis may occasionally give rise to pneumonia of this type and the same is probably true of the virus of lymphogranuloma inguinale. Even accepting the two latter viruses as occasional causes of atypical pneumonia, this would only account for, at the most, one-fifth of all cases and the precise aetiology of the remainder is as yet unknown. Those worthy of consideration in this respect are: the virus of Weir and Horsfall,[1] which produces pneumonia in the mongoose but fails to infect other experimental animals including the ferret, mouse, rabbit, guinea-pig and monkey; the virus isolated by Eaton and his associates[2] by the inoculation of cotton rats; and the feline pneumonia viruses of Blake, Howard and Tatlock,[3] and of Baker[4]; the last appears to be related to the psittacosis group. What part, if any, these viruses play in the causation of atypical pneumonia has still to be determined.

"*Cold auto-agglutination*" *reaction.*—It has been shown that in cases of primary atypical pneumonia the serum may agglutinate human erythrocytes of the blood group O at low temperatures. This reaction is absent, unless in low dilutions of serum, in other types of pneumonia, other infections of the respiratory passages and normal individuals, and has been suggested as a means of confirming a diagnosis of atypical pneumonia. The reaction, however, tends to be late in its appearance during the illness.

The test can be carried out quantitatively by preparing doubling dilutions of serum from 1 in 10 to 1 in 2560 and adding to each washed group O human red cells. The mixtures are placed in a refrigerator at 0°–4° C. for one hour when readings of agglutination are made. As the agglutinin is readily absorbed by erythrocytes at low temperature, the serum should be separated from the blood specimen at a temperature above 20° C.. A titre of 1 in 80 (in terms of the final dilution of serum after addition of red cells) might be considered significant but much higher titres have been recorded. The reaction, however, requires further study from the diagnostic standpoint. (See Meiklejohn, G., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54**, 181.)

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[1] See *J. Exp. Med.*, 1940, **72**, 595; [2] *Science*, 1942, **96**, 518; [3] *Yale J. Biol. Med.*, 1942, **15**, 139; [4] *Science*, 1942, **96**, 475.

Foot-and-Mouth Disease

Foot-and-mouth disease is an extremely infectious epizootic condition of cattle, and other cloven-hoofed animals, characterised by the occurrence of vesicles on the feet and in the mouth, along with constitutional symptoms of infection. It is occasionally communicated to man by contact and through the agency of milk. The disease can be transmitted experimentally by the inoculation of fluid from the vesicles even after filtration through the finest earthenware filters. No visible or cultivable organisms have been found aetiologically associated with the disease, and the infection is due to a filterable virus. The virus is present also in the saliva, milk and excreta of infected animals, and it has been thought that recovered animals may carry the infection for considerable periods, but this is doubtful. The size of the virus, which is one of the smallest, is 8 to 12 mμ. It has been cultivated in tissue culture by using the pads, lips, tongue and hairy skin of embryo guinea-pigs. The disease can be produced experimentally in guinea-pigs by cutaneous inoculation in the hairless pads of the feet. Within four days pyrexia results, and an inflammatory condition of the foot, with a vesicular eruption like that of the natural disease. Secondary vesicles occur on the other feet and in the mouth. The animal usually recovers and is thereafter immune to further infection. The virus is present in the circulating blood as well as in the vesicular lesions. If slowly dried the virus retains its viability for considerable periods and the disease may be transmitted by certain vehicles of infection—*e.g.* fodder—as well as by contact. It is highly susceptible to heat, and is rapidly killed at 55° C.. Three immunological types of the virus have been recognised—namely, O, A and C. Animals recovered from one type are not immune against another type, although a slight degree of cross-immunity does exist. Active immunity has been produced by the injection of formolised virus but no method of immunisation has yet proved fully satisfactory for general application. Immunity following recovery is apparently due to a viricidal antibody present in the blood serum.

Vesicular stomatitis of horses resembles foot-and-mouth disease in some features. The virus is, however, antigenically distinct and differs also in being larger (70–100 mμ) and in being cultivable in the chorio-allantoic membrane of the chick.

Rift Valley Fever

This disease, also known as enzootic hepatitis, was first described by Daubney and Hudson in 1931 in Kenya Colony, British East Africa, where it caused an extremely fatal epizootic amongst lambs. The ewes also were affected, but not so fatally. The native shepherds and Europeans engaged in the epizootic developed a dengue-like fever with severe back pains, while laboratory workers also became infected. The virus of Rift Valley fever will pass fine Chamberland filters, and by means of collodion membranes its size has been determined as about 30 mμ. It has been cultivated in tissue cultures of chick embryo in Tyrode's solution. In addition to being infective for sheep and man, the disease can be transmitted by blood, liver or spleen tissue to cattle, monkeys and rodents such as rats, mice, field voles, dormice and squirrels. It is not infective for birds, reptiles, rabbits or guinea-pigs. In the natural disease in the sheep there is extreme necrosis of the liver, with haemorrhages into other organs. Inclusion bodies are numerous in the liver. The serum of recovered animals neutralises the virus. The virus is resistant to phenol, and in defibrinated blood containing 0·5 per cent. phenol will retain its activity for many months in the cold. Mice can be immunised with virus inactivated by formalin. The disease is not contagious and there is evidence to suggest that it is transmitted by a mosquito, probably of the genus *Aëdes*.

Louping-Ill

This disease is an encephalomyelitis of sheep characterised by cerebellar ataxia and disorder of nervous functions. It is common in certain parts of Scotland. The virus is readily filterable through Berkefeld and coarse Chamberland filters. Its size has been estimated as 15–20 mμ. It can be cultivated in the chorio-allantoic membrane of the chick embryo and in a medium of chick embryo tissue *in vitro*. The virus can be transmitted by intracerebral injection of emulsions of infected brain and spinal cord to sheep, pigs and mice, and in the latter animal also by intranasal instillation. The virus enters the olfactory bulbs and from there spreads throughout the nervous system. The evidence shows that the tick, *Ixodes ricinus*, is the vector of the disease. Another infection of sheep, "Tick-bite fever" (possibly due to a rickettsial organism) is regarded as predisposing to the louping-ill infection.

Successful prophylaxis has been achieved with a vaccine of formolised sheep brain tissue from an animal inoculated with the virus, and also a formolised culture of the virus in a chick embryo medium.

Cases have been recorded of transmission of this virus to laboratory workers handling infective material. See also Australian encephalitis, p. 615.

Canine Distemper

Distemper is a specific infectious disease of great prevalence in young dogs and other animals, such as ferrets, silver foxes and fitches. The common manifestations are pyrexia and coryza, followed by secondary inflammation of the respiratory or alimentary system. Experimental evidence goes to show that the infective agent is a filterable virus. The disease can be transmitted experimentally to dogs and ferrets by subcutaneous inoculation with discharges from infected animals, and also with blood, serum and certain tissues—*e.g.* spleen. Filtrates of infective material are also virulent. The virus has been cultivated in the chorio-allantoic membrane of the chick.

Active immunisation of dogs as a prophylactic against distemper can be achieved by the injection of either (1) 5 c.c. of "vaccine" followed fourteen days later by an injection of 1 c.c. "virus"; or (2) 1 c.c. "virus" followed one and a half to two hours later by 10 c.c. immune serum. The "vaccine" consists of tissues from infected dogs, mesenteric glands and spleen, which are ground, emulsified and treated with formalin. The "virus" is derived from the spleen and mesenteric glands of infected ferrets. A 20 per cent. emulsion is made and centrifuged. The supernatant fluid is filled into ampoules, dried, and sealed under nitrogen. The immune serum is obtained from dogs immunised with distemper virus.

The results of these methods have been highly satisfactory in the prophylaxis of distemper in dogs.

B. bronchisepticus and other bacteria, at one time described as the causal agents of distemper, are secondary invaders.

Feline infectious enteritis is an acute disease of cats characterised by a short period of illness and a high death rate. Formolised infected spleen appears to provide a vaccine of some value.

The term "*Cat Distemper*" is sometimes used for a condition characterised by marked catarrh of the respiratory passages and a long period of illness, and although a virus is suspected as the cause, it has not yet been demonstrated.

Borna Disease of Horses

This is an infectious meningo-encephalomyelitis of horses, characterised by lesions in the central and peripheral nervous systems. After general systemic disturbances the animal becomes paralytic, and usually dies, the mortality being at least 75–80 per cent. The virus is essentially neurotropic. It may also infect cattle and sheep under natural conditions. It can be transmitted experimentally by intracerebral inoculation of these animals, and also rabbits, guinea-pigs, rats, mice, fowls and monkeys. The virus can pass Berkefeld and the coarse and medium grades of Chamberland filters. Its size is computed as 100 (85–125) $m\mu$ and it has not yet been cultivated. The microscopic lesions in the brain are perivascular and meningeal infiltrations with mononuclear cells, with also some diffuse infiltration. Similar lesions occur in the cord. The nuclei of the nerve cells contain characteristic inclusion bodies known as "Joest-Degen corpuscles," and are more numerous in the cells of the spinal ganglia. They vary in size from 3 or 4μ down to the limit of resolution, and by Mann's methyl-blue eosin stain are coloured red. The larger bodies are similar to the Negri bodies of rabies. The virus material treated with phenol and glycerol possesses immunising properties.

Equine Encephalomyelitis

Equine encephalomyelitis is a disease of horses which in recent years has been prevalent in the North American continent. It is due to a virus of which there are two distinct types; a "Western" and "Eastern" both of which can be cultivated in the chorio-allantoic membrane of the chick. The infection can be transmitted by various species of mosquitoes and human cases of the disease have been reported. Since the introduction of formolised chick embryo culture vaccines (1939) preventive inoculation of horses seems to have greatly reduced the incidence of the disease. A new antigenic type has been isolated in Venezuela which is more pathogenic to the guinea-pig than the types found in the United States.

Rinderpest

Rinderpest or cattle plague is characterised by elevation of temperature, glairy discharge from eyes and nose, ulceration of the lips and pads, abdominal pains and severe diarrhoea, followed by weakness and death, usually in four to seven days. *Post mortem* there is extensive submucous haemorrhage in the intestine, and ulceration is usually present along the whole alimentary canal. The causative organism is a filterable virus which passes Berkefeld and coarse Chamberland filters. The virus is not free in the blood stream but is present only in the leucocytes. It is rapidly destroyed by drying, by heat, and by chemicals, and if kept at room temperature. It survives for some months if infected blood is frozen solid, but is rapidly destroyed by glycerol. The virus is present in the discharges, all of which are infective, particularly that from the nose; infection is normally through a mucous surface and not by an insect carrier. Recovery results in a solid and lasting immunity. Formolised spleen pulp from infected animals confers a high degree of protection. A hyperimmune serum [1] is applied therapeutically. Injection of serum together with virulent blood is used to produce immunity in herds. Regarding the virus little is known; it has not been measured or photographed, and has not yet been cultivated.

Swine Fever

This disease was at one time thought to be due to an organism of the *Salmonella* group—*B. suipestifer*—but a filterable virus is the causal agent. In the acute disease there is a febrile septicaemic infection with haemorrhagic inflammation of the intestinal mucous membrane. In chronic cases there is a necrotic condition of the mucosa in which *Salmonella* organisms are found. The incubation period is about six days.

The natural mode of infection is by ingestion, and exceedingly minute doses of virus will cause the disease. The virus is present in the blood plasma and not in the corpuscles.

The virus passes fine filters. It is fairly resistant and survives moderate heat and freezing. It can also survive in cured hams and pickled pork, while desiccation does not destroy it. The virus is also resistant to disinfectants and glycerol, but is soon destroyed by putrefaction.

The immunity following an attack is lasting, and hyperimmune serum [1] together with infected blood has been used for immunisation. A form of vaccine which constitutes an effective and safe immunising agent is infected blood to which has been added 0·05 per cent. crystal violet with disodium phosphate to render the mixture of a certain degree of alkalinity, the preparation being then incubated at 37° C. for 14 days. The virus is apparently inactivated by the crystal violet without losing its antigenic properties.

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[1] The amount of antibodies in an animal which has recovered from the infection can be increased by repeated injections of infected blood. This method has been termed *hyperimmunisation*, and the serum of the animal is referred to as ***hyperimmune serum.***

Animal Pox Diseases

Various domestic animals are susceptible to pock-like eruptive skin lesions—namely, horses, sheep, cattle, goats and swine. Fowls suffer from an analogous disease.

Cow-pox or vaccinia has already been described (p. 618) and may affect man—*e.g.* milkers—under natural conditions, while *sheep-pox* is very closely allied. Inoculation of sheep-pox, whether in man, rabbits, cattle, etc., produces immunity to cow-pox, and in man also to smallpox.

Sheep-pox is often a very serious disease, and flocks can be immunised with cow-pox (vaccinia) virus or by an admixture of sheep-pox virus and immune serum.

Goat-pox occurs only where the animals are herded. The virus is closely allied to those of sheep-pox and cow-pox.

Swine-pox is similar to sheep-pox and cow-pox, and may have a mortality rate of 20 per cent. or more.

Horse-pox, known as " grease," affects the mouth, nose and fetlocks. It is uncommon in this country and probably arises from cow-pox.

All the animal pox diseases and smallpox are intimately related. There is marked cross-immunity amongst them. The smallpox virus inoculated into calves becomes so modified as to assume the characters of the cow-pox virus. Infection with one disease produces immunity to the remainder. It seems probable that cow-pox is the original or primordial disease which has affected other animals and man, and become modified according to the animal in which it has become parasitic.

Fowl-pox, *Roup*.—These diseases are both caused by the same virus, the relationship of which to the other pox viruses is doubtful. In the infected bird the virus gives rise to inclusion bodies (Bollinger bodies) and elementary bodies (Borrel bodies) are also demonstrable (*vide* p. 609). It can be cultivated in the chorio-allantoic membrane of the developing egg. Fowls can be immunised by the inoculation of pigeon-pox virus, which has a low pathogenicity for the fowl, but is probably a modified form of the fowl-pox virus.

BACTERIOPHAGE ACTION or TRANSMISSIBLE LYSIS

(*Twort-d'Herelle Phenomenon*)

This phenomenon was demonstrated by d'Herelle in the following way : a few drops of liquid faeces from a case of bacillary dysentery were added to a tube of broth which was incubated overnight ; filtration of the culture through an earthenware filter yielded a filtrate which, added in small quantities to a young culture of *Bacillus dysenteriae* (Shiga), produced lysis of the bacteria after a period of incubation; further, a filtrate of this lysed culture possessed a similar lytic property towards a fresh culture, and the lytic property was thus shown to be indefinitely transmissible from culture to culture. If a filtrate containing the lytic agent or "bacteriophage" is added to a young broth culture and this is used for inoculating a plate of medium, the resulting growth shows clear circular plaques where the phage[1] is acting: these have been spoken of as "colonies of bacteriophage." It has been suggested by d'Herelle that the effect is due to an ultramicroscopic organism (*Bacteriophage*) which is parasitic and destructive towards the particular bacteria, propagating itself at their expense. According to this view it belongs to the same category as the filterable viruses and undoubtedly a bacteriophage exhibits a close analogy to the viruses. The particulate nature of phages has been demonstrated by the same methods as those applied to the viruses (*vide* p. 596). They vary, however, in size from about 75 mμ to 10 mμ. The lytic principle has considerable resistance to physical and chemical agents; it may withstand heating at 75° C. for half-an-hour, and resists drying for long periods. It can be propagated only in living and growing cultures. No final statement can be made as to whether the phenomenon is due to a living organism or not. It is believed by some workers that the lytic agent is an enzyme developed by the bacteria themselves and that the action of the enzyme on the bacteria brings about the formation of more enzyme so that the phage undergoes apparent multiplication. The question of the unity or multiplicity of phages is still unsettled: a phage which is active for one organism may lyse related bacteria and may apparently be "adapted" to entirely different organisms. It is usually observed that, when a culture is lysed by phage, individual organisms prove resistant, and in the course of time yield a "secondary growth" which represents a strain resistant to the phage. This strain, though insusceptible to phage-lysis, may carry the phage and is designated "lysogenic" in view of the fact that culture-filtrates from it may lyse a susceptible strain. Resistant strains derived from a culture acted on by phage often constitute variants of the parent organism, and it is now recognised that phage is a powerful agency in bringing about bacterial variation. With regard to the distribution of phages, it may be said in general that a phage active towards a particular species is commonly found associated with the organism or is present in the same environment, but exceptions to this rule may be observed. Bacteriophage has an antigenic individuality apart from the associated organism and immunisation with phage-containing filtrates yields an antiserum which inhibits the action of the phage.

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[1] The term "bacteriophage" is generally abbreviated to "phage."

References to the practical applications of bacteriophages have been made in earlier chapters (*vide* pp. 433, 458).

Isolation of Bacteriophage.

To illustrate the procedure for the demonstration and isolation of a bacteriophage, the methods applicable for obtaining phages for enteric and dysentery bacilli from faeces, sewage and water may be taken.

Faeces and Sewage.—A relatively large amount of the material (*e.g.* about 5 grams of faeces or 10 c.c. of sewage) is thoroughly disintegrated and emulsified in 50 c.c. of broth (pH 7·6) contained in a 200 c.c. flask, so that conditions are sufficiently aerobic. The flask is incubated for 12 to 24 hours at 37° C. and then filtered through a Seitz disk (pp. 88, 645 and 649). It is preferable, however, to carry out "phage-enrichment" by adding to the broth a few drops of a young broth culture, of the type of organism for which a phage is sought, such as the Flexner type of dysentery bacillus. The flask is incubated and the contents are filtered as above.

To demonstrate the phage in the filtrate obtained by either of these methods an agar plate is stroked with large loopfuls of a young broth culture of the particular organism, e.g. *B. dysenteriae* Flexner, so as to yield fairly broad bands of growth on the medium; after the inoculum has dried on the medium drops of the filtrate are superimposed on the strokes. The plates are incubated overnight and the presence of phage is denoted by a clear area where the filtrate has been placed on the stroked inocula, the organisms in these areas having been lysed by the phage.

Having demonstrated the presence of phage in the filtrate, it can be maintained by adding the original filtrate to broth inoculated with the appropriate organisms, a filtrate again being prepared after incubation; and the phage can be propagated serially in the same way.

In demonstrating a phage towards the particular organism, some information may also be obtained regarding its effect on allied species or types by stroking broth cultures of these on the plate in parallel with the test organism and superimposing drops of the filtrate on them; a single plate may suffice for testing simultaneously phage action on several organisms.

The clear areas of lysis may be due to a mixture of phages of different types and if a pure phage is required its isolation may be effected by the following method. A series of decimal dilutions (in broth) of the filtrate is prepared each of which is inoculated with the particular organism; for this purpose a fairly dense suspension from an agar slope culture is made, and sufficient of this is added to the diluted filtrate to yield a slight but distinct turbidity. A few drops of each dilution so inoculated are then spread on an agar plate with a glass spreader (p. 168) so that most of the surface of the medium is inoculated uniformly. (A confluent growth over the surface of the plate is aimed at.) After incubation of the plate small discrete, well-separated "plaques" will be observed in the case of certain dilutions of filtrate. (When the phage is in too high a concentration the plaques are not sufficiently discrete). A plate representing a dilution with good separation of plaques is selected, and with a small inoculating loop single plaques are "picked-off" into tubes of broth inoculated with the test organisms, and after incubation these cultures are filtered. To ensure absolute purity it may be necessary to repeat the isolation procedure several times, preparing decimal dilutions, plating, and picking-off discrete plaques.

Water.—200 c.c. of water are added to a flask containing 100 c.c. triple strength broth. This is inoculated with 2 c.c. of a young broth culture of the organism for which the phage is sought, and the procedure is thereafter the same as that described above. Larger quantities of water can be examined by multiplying the number of flasks and 200 c.c. volumes of water tested.

METHODS OF FILTRATION

Reference has already been made in Chapter IV to the various forms of bacterial filters, and the principles of filtration in relation to the study of the viruses have been considered above.

The various types of filters used are:

(1) Earthenware candles, *e.g.* Berkefeld, Chamberland, Doulton, Mandler.

(2) Asbestos disks, *e.g.* Seitz.

(3) Glass filters made of finely ground glass fused sufficiently to make the small particles adhere, as in the sintered glass filters of Schott and Genossen of Jena, and now made in England.

(4) Collodion membranes for estimation of the size of virus particles.

(5) Sand and paper pulp filters for removing larger particles and clarifying exudates, tissue emulsions, etc..

As fluids do not readily pass through filters by gravity, it is necessary to use positive or negative pressure. Suction is the most convenient method of filtration, the fluid being drawn through the filter into a sterile container, usually a " filtering flask " which is a conical flask of thick glass with a side-arm.

When using a filter of the Berkefeld type the earthenware " candle " is fitted by means of a screw and washers into a cylindrical glass mantle, and the metal tube of the filter passes through a rubber stopper which is fitted into the neck of the flask. The side-arm of the flask is connected with an exhaust pump by pressure tubing. The fluid is poured into the mantle and after filtration is collected into the flask. The necessary suction is obtained by the usual form of water pump or by a mechanical air pump (*e.g.* Geryk). The negative pressure is estimated by means of an attached mercury or other type of manometer.

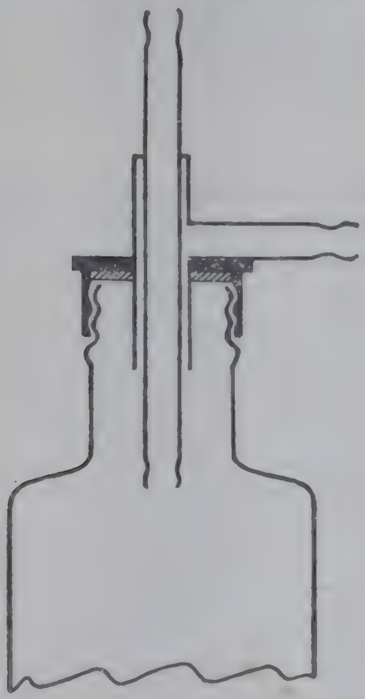
Similarly, when using a Seitz filter the metal tube may be inserted into a rubber bung which fits into a filtering flask.

A disadvantage of the filtering flask is that the filtered fluid has to be transferred later to another container and where it is desired to store filtered fluids, *e.g.* serum or culture media, contamination may occur in the process. It has also been our experience that rubber bungs are not resilient after one autoclaving and do not again fit satisfactorily so that it is necessary to tie the bung to the filter flask and seal the joints with wax.

As an alternative to a filtering flask a simple fitting attached to a screw-top bottle can be recommended. It consists of a straight piece of metal tubing, 6–7 mm. external diameter, surrounded by a wider piece of tubing to which is fitted a side-arm. The tubes are fitted into a metal screw-cap furnished with a washer to secure an air-tight joint (see Figure). The fitting is made of brass and chromium plated. Any of the screw-capped bottles can be used according to the amount of fluid to be filtered. As several sizes of bottles may fit one size of screw-cap (*vide* p. 97), a few different sizes of cap will cover a range from a few c.c. up to 4 litres. The filter employed, *e.g.* Berkefeld, Seitz, sintered glass, or paper pulp, is connected to the top of the fitting by pressure rubber tubing.

If it is desired that the fluid should not come in contact with metal, a piece of glass tubing is passed through the central metal tube, and is held in position and an air-tight joint secured by means of a piece of rubber tubing at each end of the metal tube. A wide central tube is necessary for this. Alternatively an attachment without the central metal tube can be used; the glass tubing takes the place of the central metal tube and is secured to the upper end of the wider tube by a short length of rubber tubing which grips both.

One of the advantages of the metal screw-cap fitting is that when the filtrate has to be stored, *e.g.* toxin, serum, etc., it need not be removed from the container. An ordinary screw-cap for the bottle is wrapped in kraft paper and sterilised with the remainder of the apparatus. After filtration the filter and screw attachment are removed, the ordinary cap is taken from its sterile wrapper and screwed on. Where the filtrate is to be kept for some time a viskap (*vide* p. 185) over the screw-cap is recommended to exclude dust or obviate unauthorised opening.



GENERAL TECHNIQUE OF FILTRATION

The smallest negative pressure that produces satisfactory filtration should be used, commencing with a small pressure and gradually increasing as filtration proceeds. It should be noted, however, that the time of filtration should not be prolonged as with a slight pressure over a prolonged time small motile flexible organisms such as spirochaetes and slender vibrios may pass through the filter. A high negative pressure must be avoided as small particles are rapidly forced into the pores of the filter, thus preventing further filtration. For ordinary purposes a negative pressure of 100–200 mm. of mercury is usually sufficient.

Berkefeld Filters

Before setting up the filter it should be tested for gross leaks by passing air through it under pressure while the candle is under water. If large bubbles of air escape the filter is unsuitable. Faults usually occur at the junction of the candle and metal holder, but cracks may be seen in the earthenware portion.

Water should be drawn through the filter both ways before it is put into use.

The procedure with the small size ($2\frac{1}{2}$ in. × $\frac{1}{2}$ in.) when using a filtering flask is as follows. In order to secure the maximum amount of filtrate a test tube slightly wider and longer than the candle should be inverted over it. This ensures that the whole of the candle is covered with fluid almost to the end of the filtration. Before sterilisation, the glass mantle, the candle and covering test-tube are loosely assembled without tightening up the screw and washers. The open end of the glass cylinder is plugged with cotton-wool, and the metal tube of the candle is inserted into the rubber stopper which fits the filter flask. The whole is wrapped in kraft paper and sterilised by steaming or autoclaving. The filter flask is plugged with cotton-wool, over which is tied a piece of kraft paper, and a small air filter, as figured on p. 123, is attached to the side-arm by means of rubber tubing. The flask is sterilised in the steamer or autoclave. It is advisable, where several filters and flasks are kept sterilised, to have corresponding numbers on the filters and flasks, to ensure the rubber stopper attached to the filter candle being used with the appropriate flask. When filtration is to be carried out the filter is unwrapped, the bung inserted into the flask and the washers are tightened up. The air filter is connected to the vacuum supply, fluid is poured into the mantle and gentle suction commenced. After filtration the filter and rubber bung are removed and the filtrate is transferred to a sterile container by means of a pipette.

When using the screw attachment with a screw-cap bottle, the mantle, filter and covering tube are loosely assembled. The metal tube of the filter is connected by pressure tubing with the screw attachment, to the side-arm of which is attached an air filter (Fig. p. 123) by means of rubber tubing. The screw-capped bottle is *loosely* inserted into the screw fitting.

The whole is wrapped in kraft paper or a piece of cloth and sterilised in the steamer or autoclave. An ordinary cap wrapped in kraft paper is sterilised with the outfit.

For filtration the apparatus is unwrapped, the bottle screwed tightly on, the filter and bottle are held securely in a retort stand and the air filter is connected with the vacuum supply. After filtration the bottle is unscrewed from the screw attachment and the ordinary cap screwed on.

Large Berkefeld filters are fitted into tall narrow cylinders, and the outlet-end connected with rubber tubing to a glass tube which passes through the rubber bung of a large filtering flask, or the filter may be connected to the top of a screw attachment which fits a narrow-mouth one-gallon bottle. The fluid to be filtered should always cover the porous part of the candle.

After use the filters should be brushed with a stiff nail brush and then boiled in distilled water. Before sterilising again, distilled water should be run through them to show that they are pervious. When the pores of earthenware or porcelain filters become clogged with organic matter they should be heated to redness in a muffle furnace and allowed to cool slowly.

Chamberland Filters

The large sizes are used in the same manner as the larger Berkefeld filters.

The smaller candles, such as sizes 6 and 7 of Nos. L_{1a}, L_2 and L_3, are fitted into a rubber bung so that the open end of the candle projects just above the top of the bung and the candle itself hangs down in the filtering flask. The fluid to be filtered is placed in the open end of the candle, and filtration occurs from the inside to the outside of the filter.

Seitz Filters

The filter is loosely assembled with the asbestos disk in position and the delivery tube passed through a rubber bung when a filtering flask is used. The whole is wrapped in kraft paper and sterilised in the steamer or autoclave. The filtering flask is plugged and fitted with an air filter as described under Berkefeld filters.

When a screw fitting is used, the filter is assembled with an asbestos disk and the delivery tube connected by pressure tubing with the screw attachment previously described, to the side-arm of which is attached an air filter. (When small amounts of sterile fluid have repeatedly to be removed, *e.g.*

sugars, serum, etc., the apparatus figured on p. 123 may be used.) The bottle is *loosely* screwed into the screw attachment, the whole wrapped in kraft paper and sterilised in the steamer or autoclave. When required for use the screw-cap is tightened up on the rubber washer and filtration proceeded with as above. After filtration the bottle is unscrewed and an ordinary sterile screw-cap fitted to it. The used asbestos disk is discarded. When using Seitz filters it is advisable to moisten the disk with sterile saline and then screw down tightly the upper part of the metal on the softened asbestos before pouring in the liquid to be filtered.

Sintered Glass Filters

These are used chiefly for clarifying fluids before filtration through earthenware or Seitz filters. The finest grades of these filters are comparable in porosity with the Berkefeld. They are attached to the filtering apparatus and sterilised in the same way as the Seitz filter, but care must be taken that extremes of temperature are avoided. After use they are washed with running water in the reverse direction. They should be cleaned with warm sulphuric acid to which has been added a quantity of potassium nitrate and not with sulphuric-acid-bichromate mixture.

Sand and Paper Pulp Filters

Before filtering nasal washings or other tenacious materials through Berkefeld or Chamberland filters, it is advisable to pass them through a sand and paper pulp filter. This can easily be made from a piece of glass tubing, 8 in. long by 1 in. diameter, with the lower end drawn out to a ¼-in. bore. At the bottom of the wide portion of the tube is a small perforated porcelain disk, or plug of cotton-wool, and on the top of this is placed about ½ in. of paper pulp which has been well shredded and soaked in water. This is gently pressed down, and on the top is placed about 1 in. of fine washed silver sand. On top of the sand a further ½ in. of paper pulp is placed, and the whole three layers are pressed down. The lower end of the filter fits into a bung and filtering flask or is connected by rubber tubing to the screw attachment figured on p. 647. The whole is wrapped in kraft paper and sterilised in the steamer. Filtration is carried out by suction in the usual manner.

This preliminary filtration removes coarse particles, mucus, etc., which would be liable to clog the pores of an earthenware filter.

Collodion Filters

Larger works or original papers should be consulted regarding these.[1]

Filtration of small amounts of fluid

With the smaller sizes of Berkefeld or Seitz (3 cm.) filters, a small test-tube is arranged inside the filtering flask so that the delivery tube of the filter projects into the open end of the tube and the filtered fluid is collected directly in the small tube instead of the flask itself. Similarly, with the Chamberland filter the lower end of the candle fits into a small test-tube inside the flask.

A simple but effective arrangement is to use a thick test-tube about 6 in. × 1 in. with a delivery tube drawn out at the bottom and a side-arm fused in about 1 in. from the top. A small Chamberland filter is fitted with its open end just above the surface of a rubber bung which fits the test-tube. The delivery tube is fitted with pressure tubing, a screw clamp and a hooded pipette so that the filtered liquid can be taken off under sterile conditions.

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[1] See footnote on p. 600.

APPENDIX

CHAPTER I

THE ACTION OF ANTISEPTICS IN RELATION TO BACTERIAL NUTRITION: WITH PARTICULAR REFERENCE TO THE SULPHONAMIDE COMPOUNDS.

Several references have been made in the text of this book (pp. 22, 332, 333, etc.) to the results achieved by the use of sulphonamide compounds as therapeutic agents in various bacterial infections. In order to understand the mode of action of these drugs certain aspects of bacterial nutrition (a subject alluded to in Chapter IV) require further consideration.

The growth of bacteria involves the synthesis of complex substances such as proteins, carbohydrates and fats of which bacteria are constituted. Some bacteria are able to synthesise these components from simple starting materials or " building stones "—*e.g.*, proteins from ammonium salts. The necessary energy is derived by oxidative breakdown of sugars (*e.g.*, glucose). The exact mechanism of these syntheses is unknown, but the reactions involved are effected by the many enzymes of the bacterial cell.

The enzymic transformation of simple substances into the complex constituents of bacteria must involve the formation of various intermediate substances of increasing complexity. Interference with any one of these reactions may interrupt the metabolism of the organism and prevent its growth. The number and nature of the enzymes present in different bacteria is variable and it will be appreciated that some organisms, lacking enzymes possessed by others, are unable to bring about changes which are readily effected by the latter. Thus, organisms of the *B. coli* group have a very effective enzyme system and are able to utilise ammonium salts as the *sole* source of nitrogen. On the other hand, *B. typhosus* lacks the power of synthesising certain amino-acids, notably tryptophane, and will not grow unless supplied with them. Substances such as tryptophane which are essential for growth are known as " essential metabolites." These essential metabolites which cannot be synthesised by the organism itself are known as " growth factors," *e.g.*, tryptophane in the case of *B. typhosus*.

The pathogenic organisms being parasitic have more exacting nutritional requirements (owing to their lower synthetic powers) than the saprophytic organisms.

The sulphonamide drugs inhibit growth of bacteria *in vitro* and *in vivo* and the available evidence suggests that the mode of action consists in an interference with some essential metabolite or with the enzyme associated with that metabolite, [1, 2]. It has been suggested,[3] that this hypothesis may be generally applicable to the action of other substances which prevent the growth of bacteria. Such a mechanism will account for the antiseptic action of mercury salts, in which case it is probable that the mercury combines with the metabolite thus depriving the organism of an essential "building stone." Extracts of streptococci and of yeast have been shown to reverse the action of the sulphonamide compounds, *i.e.*, the extracts are antagonistic to them. In the case of the yeast extract the material responsible for this antagonistic action has been identified with *p*-aminobenzoic acid.[2] This substance is supposed to be an essential metabolite for many bacteria and it has a structural similarity with the sulphonamide compounds. In view of this structural similarity it has been postulated that the drug "competes" with the metabolite for the associated enzyme. If the sulphonamide compound is in sufficient excess the competition for the enzyme will be decided in favour of the drug, so that the enzyme will be unable to act upon sufficient quantity of the *p*-aminobenzoic acid for this stage of the metabolism of the organism to proceed, with the result that growth of the organism will cease. In other words, the sulphonamide drugs probably act by inhibiting an enzymic reaction, fundamental in the metabolism of the organism.

Studies of other growth-promoting substances and their chemical analogues have supported this theory.[4] For example it has been possible to "design" substances which will inhibit bacterial growth by substituting $—SO_3H$ or SO_2NH_2 for $—COOH$ in a growth-promoting compound; thus, pantoyltaurine is derived in this way from pantothenic acid which is necessary for the growth of *Streptococcus pyogenes*, pneumococcus and certain other organisms, and it is found that pantoyltaurine is bacteriostatic to these organisms.

The question also arises, in regard to sulphonamide-resistant strains of species which are usually inhibited by the

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[1] Fildes, P., *Lancet*, 1940, i, 955.

[2] Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

[3] Fildes, P., *Brit. J. Exp. Path.*, 1940, **21**, 67.

[4] McIlwain, H., *Brit. J. Exp. Path.*, 1940, **21**, 136; 1942, **3**, 95.

sulphonamide compounds, whether such resistance is due to the synthesis by such strains of sufficient *p*-aminobenzoic acid to reverse the effect of the sulphonamide while sensitive strains lack this property. Some evidence of this has been obtained with certain organisms but not with others, and apparently sulphonamide-resistance depends on a more complex mechanism.[1]

ANTIBIOTIC SUBSTANCES OF FUNGI AND BACTERIA WITH SPECIAL REFERENCE TO PENICILLIN

A considerable variety of such fungal and bacterial products has now been described and studied as regards their chemical nature and their bacteriostatic or bactericidal action towards pathogenic bacteria. Among these an exceedingly active product of *Penicillium notatum*, discovered by Fleming and designated *Penicillin*, is now being applied as a chemotherapeutic agent in certain bacterial infections.

It has long been recognised that some bacterial species may be antagonistic to others both in nature and in laboratory cultures. *B. pyocyaneus* was known to have such effect on various bacterial species and the active principle was designated *pyocyanase.* This product is lytic to many other bacteria and it has been found to be of lipoid nature, its activity being due to unsaturated fatty acids. This organism, however, yields another antibiotic product, *pyocyanine*, which is stated to be the blue-green pigment of the organism, and a further product has been described, *hemi-pyocyanine*, which is more active towards the pathogenic fungi than towards bacteria.

The failure of non-sporing pathogenic bacteria to survive in soil also drew attention to the antagonism of soil organisms, *e.g.* sporing aerobic bacilli, streptothrices (or actinomycetes) and fungi, to the former. Thus, plate cultures of mixtures of soil organisms along with a pathogen have often revealed this effect by the occurrence of zones of lysis or absence of growth of the pathogen round the antibiotic colonies of the soil organisms. A particular species of sporing bacillus derived from soil, *B. brevis*, has been specially studied as regards its antibiotic products. It yields a substance, *gramicidin*, which is highly bactericidal to Gram-positive bacteria such as *Staphylococcus aureus*, various streptococci and the pneumococcus. It seems to be of the nature of a polypeptide. It acts both *in vitro* and *in vivo* and its toxicity to animals is relatively low. In animals experimentally infected with a virulent Gram-positive coccus, *e.g.* pneumococcus, gramicidin has been shown to have chemotherapeutic properties. It was found later that *B. brevis* yields another product, *tyrocidin*, which is active against both Gram-positive and Gram-negative organisms.

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[1] Landy, M., *et al.*, *Science*, 1943, 265.

Gramicidin and tyrocidin, though chemically related, are quite different in their properties. Tyrocidin is lytic to bacteria whereas gramicidin is non-lytic; it is toxic to animals, and is not effective *in vivo*. (The antibiotic product of *B. brevis* originally named "*tyrothricin*" is a mixture of gramicidin and tyrocidin.) The mode of action of gramicidin is still unknown.

Antibiotic substances derived from streptothrices (or actinomycetes) are exemplified by *actinomycin* which inhibits the growth of Gram-positive bacteria to a marked degree, with a lesser activity towards Gram-negative organisms. It is also antagonistic to various fungi. It is highly toxic to animals. The streptothrix from which it has been isolated is *Actinomyces antibioticus* and is characterised by the production of a black pigment. Another substance derived from a streptothrix (*Actinomyces albus*) has been named *Actinomycetin*. It is water-soluble and protein-like. It kills bacteria by lysis and a similar product obtained from *Actinomyces violaceus* seems to be identical with lysozyme, a bacteriolytic agent described by Fleming in lachrymal and mucous secretions.

A further product of a soil actinomyces has been named *Streptothricin*: it differs from other products of actinomycetes, and seems to be an organic base formed from amino-acids and contains iron in its molecule. It acts on both Gram-positive and Gram-negative organisms, and resembles tyrocidin.

Penicillin.—This substance is active mainly against Gram-positive bacteria and its potency in inhibiting the growth of these organisms is of a very high order. Thus, a 1 in 100 million dilution of a penicillin preparation in the purest form obtainable will prevent the growth of a staphylococcus. It is bactericidal and also bacteriolytic but acts only on organisms in their active phase; in the resting state bacteria are not affected. Its properties were first applied by Fleming in the selective culture of certain Gram-negative organisms, e.g. *B. influenzae*, when mixed with Gram-positive species in pathological material. Reference to this is made on p. 498 where the original method of producing penicillin is also described (see also *Appendix*—isolation of *B. pertussis*).

Susceptible organisms.

The Gram-positive pyogenic cocci (except enterococcus).
B. welchii, *Vibrion septique*, *B. oedematiens* and *B. tetani*.
Gonococcus and Meningococcus.
B. anthracis, *Actinomyces*, and *B. diphtheriae*.

Non-susceptible or relatively non-susceptible organisms.

Coli-typhoid-dysentery groups,
B. proteus and *B. pyocyaneus.*
Pasteurella, Brucella and *Haemophilus* groups.
Enterococcus
B. tuberculosis.

The substance is present in solution in the culture fluid. It is of the nature of an organic acid containing nitrogen in its molecule but its chemical synthesis has not yet been reported. Much work has recently been done with a view to obtaining the maximum output of penicillin from cultures of the mould for purposes of large-scale production, and processes for extracting penicillin in the purest and most concentrated form have been perfected, but at present the complete data that have been so obtained regarding the production and chemistry of the substance have not been published.[1] It may be pointed out that penicillin is unstable unless preserved in a desiccated form. It is quickly inactivated by acids, alkalis, metallic salts, alcohols and oxidising agents, and is also very thermolabile. For therapeutic use the sodium or calcium salt is supplied in the form of a brown or yellow powder[2] which is readily soluble in water or physiological saline ; the solution is used for administration. Its therapeutic value depends not only on its high quantitative potency but also on its non-toxicity and the maintenance of its effects even in the presence of blood, serum or pus. It is readily absorbed after intramuscular injection, but the purified preparation at present available for therapy can also be administered intravenously without toxic reactions. It is rapidly excreted, *e.g.* in the urine, and this necessitates the injection of large and repeated doses or continuous administration, *e.g.* by intramuscular or intravenous " drip." For regulating dosage penicillin has been standardised in terms of an arbitrary unit (" Oxford Unit ") originally defined as that amount which dissolved in 50 ml. meat extract broth just inhibits completely the growth of the test strain of *Staphylococcus aureus.* At present the unit may be defined as the activity contained in a certain weight of a sample of penicillin selected as a standard of reference. A total dosage up to 2,000,000 or 3,000,000 such units may be required for the treatment of severe infections. Though this chemotherapeutic agent is specially applicable in infection due to various Gram-positive organisms, it has been found to be highly effective in the treatment of gonorrhoea and in much smaller doses than that required for staphylococcal infections. It has been used also in controlling sepsis by direct application to wounds. For this purpose the calcium salt which is more stable than the sodium salt and less hygroscopic is preferable. It has been shown that penicillin is effective in the treatment of syphilis.

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[1] Reference can be made to the Medical Research Council War Memorandum No. 12, 1944, H.M. Stationery Office, London, which includes a bibliography of selected publications on penicillin.

[2] This preparation contains probably from 10 to 20 per cent. of the pure substance.

In the treatment of cases it is essential that dosage should be so regulated as to maintain a satisfactory level of bacteriostasis in the blood and the method described on p. 677 has been designed for controlling this. It should be noted that a penicillin-destroying ferment (penicillinase) is produced by various bacteria which may also be resistant to penicillin and if a solution of penicillin becomes contaminated with such an organism its active properties may be lost.

Apart from *P. notatum* a great variety of other fungi produce antibacterial substances, and in addition to penicillin a further antibiotic product, *notatin* or " *penicillin B* " has been obtained from *P. notatum.* This acts on both Gram-positive and Gram-negative bacteria, but is inactive in the absence of glucose or other carbohydrates. It is more toxic to animals than penicillin. It is considered to be a flavo-protein.

Mention may also be made of helvolic acid, an antibiotic product of *Aspergillus fumigatus* mut. *helvola.* Its properties are in many respects like those of penicillin. Helvolic acid has been extracted in crystalline form from cultures of the mould, and its probable chemical formula is $C_{32}H_{44}O_8$. It forms salts like penicillin, and the sodium salt has been used for experimental work. Though its selective bacteriostatic effects are similar to those of penicillin, the quantitative potency is less. Unlike penicillin, it can be absorbed into the blood from the alimentary tract. It is not toxic to animals in bacteriostatic concentrations. So far chemotherapeutic experiments in animals have not yielded satisfactory results.

A further antibiotic substance derived from the mould *Penicillium patulum*, and named " patulin," has also attracted attention. It is identical with a product of *Penicillium claviforme,* which has been designated " claviformin." These substances have been isolated in crystalline form and have the chemical formula, $C_7H_6O_4$. It has been shown that claviformin is capable of abolishing the respiration of *B. coli*, but serum reduces its antibacterial effect. It is injurious to leucocytes and must be regarded as a protoplasmic poison.

CHAPTER III

FLUORESCENCE MICROSCOPY

WHEN certain materials, *e.g.* vaseline, uranium ores, or uranium glass, solutions of quinine, aesculin, and various dyes are exposed to ultra violet (U.V.) light they alter the wave-length of the invisible light and so become luminous and are said to fluoresce. If tissues or bacteria are treated with a fluorescent dye and examined under the microscope, ultra violet light being used instead of ordinary visible light, they become luminous and are seen as bright objects in a dark field. Moreover, these fluorescent dyes may have a selective action on certain tissues or bacteria which are thus readily identified and recognised.

Dyes specially suited for fluorescence microscopy are auramine, coriphosphin O, morin, thioflavin S, thiazo yellow G, berberine sulphate, fuchsin, primuline, etc.. They are used in very dilute solutions, 1 in 1000—1 in 10,000, and may be employed for double staining, *e.g.* fuchsin and coriphosphin.

For fluorescence microscopy a source of U.V. light is used, either a high pressure mercury vapour lamp or direct-current carbon arc lamp suitably enclosed. As ordinary glass absorbs a considerable amount of U.V. light (particularly the shorter wave-lengths) the condensing lenses of the lamp are made of quartz. Similarly, the microscope condenser is made of quartz, while the microscope slide is made of special glass which passes U.V. light. The visible rays from the lamp are cut out by a dense filter of " Woods glass " which allows only the invisible U.V. rays to pass. As the Woods glass also transmits very deep red and infra-red rays, the light, before it passes the glass, goes through a solution of 4 per cent. copper sulphate solution to remove these rays. The rest of the microscope, *i.e.* objectives and eye-piece, are as ordinarily used since the U.V. rays when they reach the specimen cause it to fluoresce with visible light. A special filter is placed over the eye-piece to prevent any harmful effect of U.V. rays entering the eye.

It should be noted, however, that the absence of quartz lenses and condenser should not deter anyone from undertaking fluorescence microscopy, providing a satisfactory light source is available. Glass will transmit the long-wave U.V. rays, *i.e.* of wave-length greater than 300 mμ, and good fluorescent images may be obtained. With the quartz equipment of course much more U.V. light is transmitted, but in spite of this some workers assert that for diagnostic use quartz equipment does not justify the extra expense, and the ordinary microscope condenser is sufficient, particularly if used in a completely darkened room.

Fluorescence microscopy can be applied to the examination of the tubercle bacillus by substituting a solution of auramine for carbol fuchsin in the Ziehl-Neelsen method, *i.e.* the preparation is overstained with auramine and then decolorised with acid-alcohol. The tubercle bacilli still retain the stain when the rest of the material has lost it.

METHOD.—*Staining solution.* Auramine 1 in 1000, in 5 per cent. phenol in distilled water.

Decolorising solution. Industrial alcohol containing 0·4 per cent. NaCl and 0·4 per cent. HCl.

Stain a thin smear of sputum with auramine solution for fifteen minutes. Rinse under the tap and decolorise by two treatments of ninety seconds each with the acid-alcohol. Rinse and dry.

The film is examined dry with a $\frac{1}{6}$-inch objective corrected for use without a coverslip. The tubercle bacilli are seen as luminous fluorescent organisms in a dark field. It has been claimed by some workers[1] that this technique gives a higher percentage of positive results than the ordinary Ziehl-Neelsen method.

Immersion objectives can be used but ordinary cedarwood immersion oil and practically all oils are fluorescent and only special non-fluorescent liquid paraffin, or sandal-wood oil, is permissible.

ELECTRON MICROSCOPE

The resolution, and hence the degree of magnification, depends on the wave-length of light used (see p. 61). By using electrons, which are not subject to " wave-length " limitations, very great magnification and high resolution have been obtained. The apparatus employed is, however, costly, elaborate, and technically difficult to operate. The electrons travelling at a speed comparable with the speed of light, pass through magnetic fields which act as lenses and cause the necessary convergence of the electron beams. The shadow image is received on a photographic plate or may be viewed with a fluorescent screen. The material to be examined is placed on a collodion membrane (which must not be more than 1μ thick), supported by a metal gauze screen, and placed in the path of the electrons, the whole being maintained in a high vacuum to prevent the electrons being deflected by air molecules.

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[1] Richards, O. W., Kline, E. K., and Leach, R. E., *Amer. Rev. Tuber.*, 1941, **44,** 255 ; Bogen, E., *ibid.*, 1941, **44,** 267 ; Oscarrson, P. N., *Acta Med. Scand.*, 1941, **108,** 240 ; Richards, O. W. and Miller, D. K., *Amer. Journ. Clin. Path.*, 1941, ii (Technical section), 1.

By means of the electron microscope, magnifications of as much as 100,000 diameters have been obtained and particles one hundred times smaller than those resolvable by an ordinary microscope have been photographed. (See Mudd, S. and Anderson, T. F., *Journ. Amer. Med. Assoc.*, 1944, **126,** 561 and 632.)

CHAPTER IV

CLEANING OF GLASSWARE

INSTEAD of the soap solution mentioned on p. 79 it is recommended that one of the proprietary sodium metasilicate detergents be employed. The product "Kin-Ray" made by the Reddish Chemical Co., Ltd., Globe Works, Reddish, Stockport, is satisfactory for this purpose. The glassware is boiled in a $\frac{1}{2}$–1 per cent. solution (depending on the state of the glass and hardness of the water), well rinsed in ordinary water and finally in distilled water if the domestic water supply is hard.

After treatment as above the glassware has a shine and sparkle not obtained by soap preparations.

SERUM-WATER STARCH MEDIUM

This medium does not keep well as the starch undergoes gradual hydrolysis forming glucose which is fermented by all types of diphtheria bacilli (p. 376). It is essential, therefore, to make up the starch solution only when required and add it to the serum-water medium immediately before use.

A convenient method sufficient for about two dozen small bottles (holding 3 c.c. of medium) is as follows. Weigh out 0·15 gram of soluble starch and place it in a sterile universal container or other sterile 1 oz. screw-capped bottle. Add 5 c.c. distilled water, screw on the cap and shake vigorously. Place the bottle in a small saucepan or enamel mug of water, bring to the boil, and boil for about five minutes, shaking at intervals to ensure that all the starch is in solution and the contents are homogeneous. When the starch solution is cool add 0·15 c.c. with a sterile 1 c.c. pipette, or five drops from a sterile Pasteur pipette, to each of the fermentation bottles. After the starch has been added the medium should be used within a few weeks.

DESOXYCHOLATE-CITRATE-AGAR

(for the isolation of dysentery, typhoid-paratyphoid and Salmonella organisms).

Modification by M. Hynes of Leifson's medium (p. 138)

Agar	22·5 gms.
Lab.-Lemco	5·0 ,,
Difco proteose peptone (or Evans)	5·0 ,,
Lactose	10·0 ,,
Sodium citrate	8·5 ,,
Sodium thiosulphate	8·5 ,,
Ferric citrate	1·0 ,,
Sodium desoxycholate	5·0 ,,
Neutral red (as indicator)	
Water to 1,000 c.c.	

Dissolve 20 gms. Lab.-Lemco in 200 c.c. water over the flame; make just alkaline to phenol phthalein with 50 per cent. NaOH, boil and filter. Adjust the *p*H to 7·3, make up the volume to 200 c.c. and add 20 gms. Difco proteose peptone.

Dissolve 90 gms. agar in 3,700 c.c. distilled water by one hour's steaming. Filter the agar, add the Lab.-Lemco-peptone solution and mix.

Add 5 c.c. 2 per cent. neutral red and 40 gms. lactose, and mix.

Bottle in accurate 100 c.c. lots, and sterilise by free steam for one hour and then at 5 lbs. pressure for 10 minutes.

Solution A.

Sodium citrate (A.R., $Na_3C_6H_5O_7.2H_2O$)	17 gms.
Sodium thiosulphate (A.R., $Na_2S_2O_3.5H_2O$)	17 ,,
Ferric citrate (scales)	2 ,,
Distilled water	100 c.c.

Dissolve by heat or by standing at room temperature for two days.

Solution B.

Sodium desoxycholate	10 gms.
Distilled water	100 c.c.

These solutions should be sterilised at 60° C. for 1 hour.

For use, melt 100 c.c. of the agar base, and add 5 c.c. each of solutions A and B in this order, using separate pipettes and mixing well between. Pour plates *immediately* and dry the surface.

(1) The type of protein extract and peptone used greatly affects the properties of the medium, and the products recommended should not be varied without control experiments to ensure that the performance of the medium is not impaired.

(2) The medium should be poured and cooled as soon as possible after the addition of the desoxycholate, otherwise it tends to become very soft.

(3) It is no disadvantage if a rather acid reaction of the medium causes partial precipitation of the desoxycholate. Simply rubbing a loop on the medium may cause such precipitation along its track and give a false appearance of contamination.

(4) The desoxycholate must be pure, as all the common impurities impair the efficiency of the medium.

The medium is pale and slightly opaque. Some coliform strains and particularly *B. aerogenes* grow on it, producing deep pink opaque colonies 1 to 2 mm. in diameter, and causing (by acid-production) an intense precipitation of desoxycholate in the surrounding medium. The colonies of pathogens are colourless, and by their alkaline reaction redissolve the desoxycholate, so that they are surrounded by a zone of transparent medium.

Colonies of *B. dysenteriae* (*Sonne*) are round, about 2 mm. diameter, with well-defined edges and no appearance of " roughness." They may be pale pink, or become so, on further incubation or storage. Rough variants of this organism do not grow on the medium. *B. dysenteriae* (*Flexner*) colonies are similar, but may have a narrow plane periphery surrounding a central dome. Colonies of *B. paratyphosus B* and *Salmonella* organisms are larger, 2 to 4 mm. diameter, often with a central black dot. *B. typhosus* yields a flat round colony.

Of non-pathogenic non-lactose fermenters, only *Proteus* strains grow freely ; the colony is usually glossy and more translucent than those of the pathogens ; some strains produce a central black dot. There is no tendency to spread, but the characteristic fishy odour is present.

Inoculate plates *heavily* with faeces or rectal swabs in a way that will ensure discrete colonies ; incubate for 18 to 24 hours. Re-incubation for another 24 hours is occasionally necessary if there are no non-lactose fermenting colonies present after 24 hours' incubation or if the colonies are very small.

Slide-agglutination with colonies picked directly from the plate is satisfactory provided the usual precautions are taken to obtain a fairly heavy and uniform suspension.

For fermentation reactions colonies are picked, preferably with a straight wire, into peptone water and subcultured into the appropriate sugars after 4 to 6 hours' incubation. At the same time a subculture should be plated on MacConkey's medium to test the purity of the peptone water culture.

This medium is particularly suited for the isolation of the dysentery organisms, the *Salmonella* food-poisoning group, and *B. paratyphosus B*. It is not quite so selective for *B. typhosus* though superior to MacConkey's medium.

LAKED BLOOD FOR HOYLE'S MODIFICATION OF NEILL'S MEDIUM[1]

It has been shown by Young[2] that instead of laking the blood by freezing and thawing, the use of saponin in a final dilution of 1 in 200 is more simple and convenient.

Prepare a 10 per cent. solution of saponin (white) in distilled water and sterilise in the autoclave. Use 0·5 c.c. of this solution for each 10 c.c. of blood. Place the blood in the incubator for 15 minutes, add the saponin, and invert the bottle gently several times to ensure thorough mixing, but avoiding the formation of bubbles. Replace the blood in the incubator for a further 15 minutes when it should have an "inky" black appearance. Store in the refrigerator where it will keep for several months. Use as described on p. 149.

Similarly, sodium di-octyl-sulpho-succinate (Aerosol O.T. 100 per cent.[3]) in a final dilution of 1 in 500 can be used. A 1 per cent. solution in distilled water is made and the bottle left in the 60° C. water-bath overnight to effect solution and to sterilise it. The solution is stored at room temperature. 2 c.c. is used for each 10 c.c. of blood in the manner described above.

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[1] See p. 149.

[2] Young, M. Y., *Journ. Path. Bact.*, 1942, **54**, 253.

[3] Cyanamid Products, Ltd., Shootersway, Berkhamsted, Herts.

CHAPTER V

CULTIVATION OF ANAEROBES

Use of semi-solid agar medium.

A fluid medium, *e.g.* nutrient broth is heated in boiling water and to it is added one-tenth of its bulk of melted 2 per cent. nutrient agar. On cooling a semi-solid "sloppy" medium results which can be used as it is but is usually enriched with glucose, 1–2 per cent., or other reducing agent such as thioglycollic acid 0·02 per cent., sodium thioglycollate (see Brewer's medium below) or ascorbic acid 0·1 per cent. If the semi-solid medium is tubed and kept for any length of time it should be placed in boiling water for ten minutes and allowed to cool before use.

Thioglycollate media.

It has been shown by Brewer[1] that the addition of sodium thioglycollate (0·1 per cent.) maintains the anaerobic condition which prevails for a short time after the sterilisation of culture media, and anaerobes can be grown in open tubes similar to aerobic organisms. The medium also contains 0·05 per cent. agar to prevent convection currents, glucose in amounts up to 1 per cent. (according to the organism grown), and methylene blue 0·0002 per cent. (1 in 500,000) to act as an oxidation-reduction potential indicator. The methylene blue remains decolorised except for the surface layer.

The sodium thioglycollate medium recommended by Brewer consists of :—

Pork infusion solids	1 per cent.
Peptone (thio)	1 ,,
Sodium chloride	0·5 ,,
Sodium thioglycollate . . .	0·1 ,,
Agar	0·05 ,,
Glucose	0 to 1 per cent.
Methylene blue	0·002 ,, (1 in 500,000)

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[1] Brewer, J. H., *J. Amer. Med. Assoc.*, 1940, **115**, 598.

The medium is placed in 12 c.c. amounts in 6 × ⅝ in. tubes making a column of about 7 cm. The tubes are stoppered with cotton wool and autoclaved at 120° C. for 20 minutes and stored at room temperature. The medium is inoculated in the usual way.

Any suitable infusion or digest broth with the addition of sodium thioglycollate, glucose and methylene blue in the amounts stated above will serve satisfactorily.

Use of reduced iron in media.

A convenient method of converting the usual laboratory media such as broth and peptone water for anaerobic use is by the addition of iron strips. These are cut from thin sheet iron (which is really a mild steel containing less than 0·25 per cent. carbon), 26 gauge and 25 × 3 mm. in size. The medium is heated in boiling water for ten minutes, cooled, and a sterile iron strip (conveniently sterilised by flaming) is added. The medium is inoculated and incubated in the ordinary way, the iron strip ensuring anaerobic conditions. The strips fit conveniently the small ¼ oz. (bijou) bottles commonly used for fermentation tests. Sugar reactions may be noted and tests for indole may be carried out after 24–48 hours' incubation on the anaerobes referred to on p. 509 and other organisms such as anaerobic streptococci and *Bacteroides* (p. 470). The results should be read before the heavy deposit of iron hydroxide masks the reaction.

A MODIFICATION OF McINTOSH AND FILDES' JAR

The original "jar," described in 1916, consisted of a metal tin with a "press on" lid, in the centre of which was soldered a small brass gas-tight tap, and to the under surface was fixed a folded strip of brass holding a flat envelope-shaped piece of fine brass wire gauze containing palladinised asbestos. To produce anaerobic conditions the envelope was heated, the lid replaced, hydrogen introduced as described on p. 177, and the lid sealed with plasticine. Its disadvantage was that a visible indicator of anaerobiosis within the jar (p. 176) could not be employed. To overcome this McIntosh and Fildes substituted the glass jar described on p. 175. This apparatus is costly, however, and Hudson[1] has introduced a modification of the original tin whereby an external indicator tube shows whether the anaerobiosis is effective. A 3-inch length of compo-metal gas tubing (about $\frac{3}{16}$ inch external diameter) is bent at right angles for an inch of its length and this end is inserted into a hole punched in the upper part of the tin, and soldered in position to make a gas-tight joint. To the portion of tubing outside the tin is attached a 2-inch length of pressure rubber tubing. A piece of 6 mm. glass tubing is sealed at one end, and the indicator solution added (about 0·5–1·0 c.c.). A constriction is made above the liquid (to avoid spilling) and the open end is attached to the pressure tubing. The indicator solution consists of 1 per cent. glucose in Hartley's broth (*p*H 8·5–9·0), coloured deeply with methylene blue to which is added as preservative 0·01 per cent. merthiolate or 0·01 per cent. phenyl mercuric nitrate. When in use, the indicator should become decolorised after about two to three hours incubation at 37° C.. The indicator tube is left in place and used repeatedly. When the colour change becomes too small a tube of fresh indicator solution can easily be fitted. This is a cheap and effective method of anaerobic cultivation.

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[1] Hudson, R. E. B., *Brit. J. Exp. Path.*, 1941, **22**, 305.

THE USE OF *P*-AMINOBENZOIC ACID IN CULTURE MEDIA

In cases treated with sulphonamide compounds, there may be enough drug in the blood stream to prevent the growth of bacteria when blood culture is carried out. As the drug competes with the organism for *p*-aminobenzoic acid (*vide* p. 653), the addition of the latter substance to the blood culture will prevent the bacteriostatic action of the sulphonamide.

p-aminobenzoic acid is added to the broth in the proportion of 5 mgms. per 100 c.c. before the blood culture bottles are made up. It is quite stable and withstands autoclaving.

As sulphonamide compounds are now widely used, the addition of *p*-aminobenzoic acid in the above concentration will be found valuable not only in blood culture but in the media used for the isolation of pathogenic cocci. Even if no sulphonamide has been administered *p*-aminobenzoic acid improves the nutritive qualities of the medium.

RAYNER'S METHOD FOR THE PRESERVATION OF CULTURES AND SERA BY DRYING[1]

This is a development of the methods of Craigie and Hartley (p. 267) in which it is essential that the material be rapidly frozen when the vacuum is applied and that the drying takes place from the frozen state. With small quantities of liquid, however, the container (ampoule or tube) absorbs so much heat that it is difficult if not impossible to freeze the material by its own rapidity of evaporation. This is overcome by placing the culture (or serum) on thin waterproof cellophane.

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[1] Quoted from Rayner, A. G., *J. Path. and Bact.*, 1943, **55**, 373.

Cultures to be dried should be grown overnight in digest broth to which a few drops of horse blood have been added. A 4-inch Petri dish is fitted at the bottom with a disk of white filter paper. On the surface are placed small pieces of waterproof cellophane $\frac{2}{1000}$ inch thick and approximately $\frac{3}{8}$ inch square. About twenty-four can be placed on the filter paper without touching or overlapping. The dish and its contents are then sterilised in the hot air oven, the lid of the dish being kept slightly open by means of a piece of wooden swab stick cut to fit the inside of the lid. By means of a capillary pipette a single drop of the blood-broth culture is dropped in the centre of each cellophane square. (The end of the pipette must not touch the cellophane.) The dish with the lid slightly open is placed over calcium chloride in a 10-inch desiccator which is exhausted by means of a Hyvac pump. The cultures quickly freeze and dry within twenty minutes, although they are usually left overnight in the exhausted desiccator.

For preservation, each cellophane square with the dried culture is sealed *in vacuo* in a glass container. The pieces of cellophane are picked up with sterile forceps and dropped into sterile thin chemical test-tubes $5 \times \frac{1}{2}$ inch and the cotton-wool plugs replaced. The tubes are then constricted in the middle by a blowpipe flame to about $\frac{1}{16}$ inch diameter. The tubes are next placed in the desiccator, which is again exhausted and left overnight. To seal the tubes the cotton-wool plug is set alight and pushed down the tube. A soft rubber bung, with about 2 inches of 5-mm. glass tubing through it, is fitted into the tube. The glass tubing is connected to a Hyvac pump and when a vacuum is obtained the tube is sealed at the constriction in the blowpipe flame. It is possible to dry cultures of the gonococcus, meningococcus and *Haemophilus pertussis* successfully in this way.

For larger quantities of material, *e.g.* agglutinating sera and guinea-pig serum for complement, disks of waterproof cellophane $2\frac{3}{4}$ inches in diameter are placed over upturned lids of approximately 2 inches diameter, *e.g.* the lids of 2 ounces waxed cardboard sputum cartons. The disks are sterilised individually in Petri dishes in the hot air oven and placed on the waxed cardboard lids by means of sterile forceps. The serum (plain, or salted as in Hartley's method) is pipetted in 1–5 c.c.

amounts on to the cellophane disks. These are stacked in the desiccator, which is exhausted by means of the Hyvac pump. The serum rapidly freezes solid and dries in a short time, but is left overnight in the desiccator. The dried serum is detached quite easily by crumpling the cellophane, and is then placed in sterile $6 \times \frac{5}{8}$ inch test-tubes. These are constricted, again desiccated, and sealed with a vacuum as described above. The cellophane disks can be used again.

CHAPTER VI

MODIFICATION OF THE ZIEHL-NEELSEN METHOD FOR STAINING THE TUBERCLE BACILLUS

INSTEAD of employing 20 per cent. sulphuric acid as a decolorising agent (p. 205), 3 per cent. hydrochloric acid in 95 per cent. alcohol (industrial methylated spirit) may be used. The necessity for subsequent treatment with alcohol as in the original method is obviated. The time of decolorisation is longer with the acid-alcohol than with sulphuric acid, but the former is much less corrosive and more convenient to make up and employ, while its use definitely excludes organisms which are acid-fast but not alcohol-fast.

" POLYRIC " IMMERSION OIL

An improved immersion oil termed " Polyric oil " [1] is made by mixing together one part of a liquid synthetic resin, polymethylstyrene [2] (Refractive Index about 1·583) with two parts of good quality castor oil (R.I. 1·480).

The two fluids are warmed to 60° C. to render mixing more easy and any air absorbed during the shaking must be removed before the R.I. of the mixture, which should be 1·515 at 20° C., is determined.

Polyric oil is a clear colourless fluid with a slight odour. It does not dry on the objective or slide on which it may remain many months without any sign of drying or hardening, and does not become viscous or cloudy in cold weather. It is soluble in and easily removed by xylol. It is easily prepared and is a cheap and efficient substitute for cedar wood immersion oil.

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[1] For details of preparation see McCartney, J. E., *J. Path. and Bact.*, 1944, **56**, 265.

[2] Obtainable from B.X. Plastics, Ltd., Larkswood Works, Higham Station Avenue, South Chingford, London, E.4. (Purpose for which it is required should be stated.)

CHAPTER VIII

THE KAHN VERIFICATION TEST [1]

(Sero-diagnosis of syphilis)

THIS test was introduced by Kahn with the object of ascertaining whether weak or doubtful reactions obtained by his standard flocculation test are non-specific or definitely significant of syphilitic infection. From studies of "false positive" reactions he concluded that a positive result may in some cases be related to biological changes apart from syphilitic infection, but that this non-specific reaction can often be differentiated from the true syphilis reaction by the occurrence of a stronger effect at 1° C. than at 37° C., or the absence of flocculation at the higher temperature. He has called this the "general biologic type" of reaction and regards it as non-syphilitic, the reaction in the syphilitic case being usually stronger at 37° C. than at lower temperatures.

In carrying out such comparative tests at different temperatures the reagents before mixing must be adjusted to the particular temperature. Thus, for the test at 37° C. pipettes, tubes and racks are placed in a 37° C. water bath for fifteen minutes before performance of the test, and likewise the diluted antigen, serum and saline are similarly kept at 37° C. before the mixtures are made; further at all stages of the test the required temperature is maintained as far as possible. In carrying out the test at the low temperature an ice-water bath can be used in the same way as the 37° C. water bath, the working temperature being about 2° C. Otherwise the test is performed as in the standard procedure (*vide* p. 280).

The various types of comparative result are illustrated in the following table:—

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[1] Kahn, R. L., *J. Lab. and Clin. Med.*, 1940, **26,** 139; and *Arch. Dermat. and Syph.*, 1940, **41,** 817; Beveridge, W. J. M., *Edin. Med. J.*, 1943, **50,** 344.

At 37° C.	At Room temperature.	At 2° C.	Classification of result.
++++ or +++ ++	++ —	+ or — —	" Syphilitic "
+ or — —	++ —	++++ or +++ ++	" General Biologic "
++ — ++	++ + —	++ — ++	" Inconclusive "
—	—	—	Negative

It may be noted that the application of this " verification " test has shown that the sensitivity of the syphilitic reaction is increased at 37° C. as compared with room temperature, *i.e.* the temperature of the standard test.

The comparative test at 37° C. and 2° C. is of value when the usual Kahn reaction is weak or doubtful or when there is some discrepancy between the serological result and the clinical findings. It is of similar value when other syphilis serum tests also give a weak or doubtful result. Of course it must be recognised that in a proportion of such cases even the " verification " test is inconclusive (see Table).

RICHARDSON'S METHOD FOR PRESERVING LIQUID COMPLEMENT-SERUM

Preservation of liquid complement-serum in hypertonic salt solution is effective provided the *p*H is adjusted to 6–6·4 (Richardson, 1941).[1] A convenient method, employing borate-buffer-sorbitol for control of *p*H, is now described (Richardson. 1944).[1]

Two stock solutions, which keep indefinitely, are used :—

(*A*) Boric acid (H_3BO_3) 0·93 gram, borax ($Na_2B_4O_7 \cdot 10H_2O$) 2·29 grams, and sorbitol ($C_6H_{14}O_6 \cdot \frac{1}{2}H_2O$) 11·47 grams are dissolved in and made up to 100 c.c. with saturated NaCl solution. The *resulting* molar concentrations (see note 2) are : 0·27 *M* boric acid, 0·12 *M* sodium borate, 0·6 *M* sorbitol in saturated sodium chloride.

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[1] Richardson, G. M., *Lancet*, 1941, ii, 696 ; and Personal communication, 1944.

(*B*) Borax 0·57 gram and sodium azide (NaN_3) 0·81 gram are dissolved in and made up to 100 c.c. with saturated NaCl solution. The *resulting* molar concentrations (see note 2) are: 0·03 *M* boric acid, 0·03 *M* sodium borate, 0·125 *M* sodium azide in saturated sodium chloride.

To preserve complement-serum, mix 8 parts of serum with 1 part of Solution *B*, followed by 1 part of Solution *A*. This treated serum keeps very well even at room temperature. At 0° to 3° C. loss of titre is not noticeable until after six to nine months. The mixture contains 0·03 *M* boric acid, 0·015 *M* sodium borate, 0·06 *M* sorbitol, and 0·0125 *M* sodium azide.

For use as 1 in 10 complement, 1 part of preserved serum is diluted with 7 parts of distilled water. Any further dilution from the 1 in 10 mixture is made with saline. Diluted serum should not be kept more than an hour or two. According to Richardson no case of faulty behaviour in the Wassermann reaction attributable to preserved serum has come to notice.

Notes on the method.

(1) The amount of sodium chloride in a saturated solution (about 31·7 grams per 100 c.c.) is practically unaffected by changes in room temperature.

(2) Borax in aqueous solution gives an equimolar mixture of sodium borate (NaH_2BO_3 or $NaBO_2$) and boric acid (H_3BO_3).

(3) Solution *A* has a *p*H of about 4·5, acidity being due to a sorbitol-boric-acid complex. It will bring the *p*H of preserved serum automatically within the desired range. When diluted, the complex dissociates and the acidity disappears.

(4) Sodium azide is a poison with similar physiological action to sodium cyanide, and solutions containing it must be handled with necessary care. If acidified, it liberates hydrozoic acid, which is equally poisonous *and volatile*. Hence a little borax is used in Solution *B* as an alkaline buffer to minimise chance volatilisation. Solution *B* should be added first to serum, and never mixed with Solution *A* (acid) in absence of serum.

(5) Though sodium azide is inert, metallic azides are detonators; it is advised that the sodium salt be handled with non-metallic spatulas.

(6) Of the ingredients, only sodium azide causes any salting out of sodium chloride from the saturated solution. Sodium chloride may thus precipitate from Solution *B*, and if desired may be decanted or filtered off.

(7) The chief cause encountered for "feeble complement" has been the presence of inhibitory quantities of zinc or similar metallic salt in the sodium chloride used for saline. Zinc and other metals may be detected by giving a white

opalescence with sodium diethyl-dithio-carbamate (B.D.H. reagents for delicate analysis). A complement titration in presence of 0·01 per cent. of this reagent (freshly dissolved) will establish whether the amount of zinc present is dangerous. The safe limit is about 0·002 per cent. zinc in sodium chloride (Richardson, 1944). Analar specifications do not at present limit the presence of zinc.

CHAPTER IX

BACTERIOLOGICAL EXAMINATION OF AIR AND AIR DISINFECTION

In the past the procedure frequently adopted for determining the relative number and types of microorganisms present in air has been to expose open plates of culture medium for given periods of time. A count of the colonies after incubation of the plates yields an approximate estimate of the number of organisms present, and if blood-agar is used the occurrence in the air of haemolytic and *viridans* streptococci can be determined. This method has proved valuable in demonstrating the presence of haemolytic streptococci in the air and dust of hospital wards containing cases of streptococcal infection, *e.g.* scarlatina. Such findings have also thrown light on cross-infection in hospitals.

It is recognised, however, that this simple method of exposing plates has certain limitations as a means of studying the bacteriology of air, and more elaborate procedures have been adopted. A technique introduced by Bourdillon, Lidwell and Thomas (*J. Hygiene*, 1941, **41**, 197) involves the use of a special instrument, the "slit sampler," by which a known volume of air is directed on to a plate through a slit of given size, the plate being mechanically rotated so that the organisms are evenly distributed over it. This method has proved valuable in the qualitative and quantitative study of air bacteria. It may be added that 5 per cent. blood-agar containing 1 : 1,000,000 crystal violet which allows streptococci to grow while inhibiting most air and dust organisms is useful for demonstrating the former as indices of contamination of air from the respiratory passages.

Recently much attention has been paid to the possibility of disinfecting the air of occupied premises, *e.g.* hospital wards, schools, cinemas, etc., so as to reduce the spread of infectious diseases communicated by droplet infection and by dust. Hypochlorous acid and certain other antiseptic chemicals have been advocated for the purpose on the basis of experimental observations with these substances. Irradiation of the air by ultra-violet light has also been shown to be an effective means of controlling bacterial contamination of the air. For this purpose mercury vapour lamps are used with the rays directed upwards, and the air of the room is circulated through the irradiated zone by means of a small electrically driven fan incorporated in the lamp assembly. This subject has been critically reviewed in the following article which may be consulted for further information: Hollaender, A., *Amer. Journ. Pub. Health*, 1943, **33,** 980.

Spraying of the air with a hypochlorite solution has appeared to combine easy practicability with a definite efficiency in reducing the bacterial content of the air. For convenience one of the proprietary hypochlorite solutions, such as "chloros" (diluted about ten times with tap water to form a 1 per cent. solution of hypochlorite) may be used; suspensions of bleaching powder have also proved satisfactory. The spraying can be effected by means of a suitable "flit" gun. To regulate the quantity used the number of c.c. delivered for a certain number of strokes of the gun can be determined. For information on the technical details of this method and its theoretical and experimental basis reference should be made to a paper by S. W. Challinor (*Journ. Hygiene*, 1943, **43**, 16) which also includes a review of the literature. Challinor has estimated that a single spraying with 11 c.c. of a 1 per cent. solution of sodium hypochlorite per 1,000 cubic feet of air, *i.e.* 0·4 parts per million, can produce approximately a 33 per cent. reduction of the *total* bacterial content of the air of a crowded room, and that repeated spraying will cause substantially effective disinfection. It has been shown that the duration of activity of a single spraying is short (about fifteen to twenty minutes) and repeated or continuous spraying is therefore necessary for maximum and sustained action. It must be remembered that efficiency depends on relative humidity which should be high for full antiseptic action. The relative humidity of the air of crowded premises is usually high, thus favouring the action of hypochlorites.

Another air-disinfectant which merits consideration is propylene glycol. It is more effective as a vapour than in the form of a spray. This can be attained by some form of slow heat vaporisation which also permits of a continuous effect, since the action of the glycol, like that of hypochlorite, is transient.

It has been shown by S. W. Challinor and J. P. Duguid ("Propylene glycol as an air disinfectant," I and II, *Edin. Med. Journn.*, 1944, **51**, 280 and 388) that ½ part per million *per hour* (0·5 c.c. liquid glycol vaporised per 1,000,000 c.c. air *per hour*) can produce approximately a 90 per cent. reduction of air contamination due to *nasopharyngeal* organisms in a crowded room. This rate of dosage does not cause misting of the air by the vapour. In order to reduce effectively the *total* bacterial content of the air of a crowded room, a greater amount (about twice as much) of the glycol is required, and this higher rate of dosage appears to be a little below that which causes misting of the air. Humidity is an important factor but, in contrast to the action of hypochlorites, the optimum effect of the glycol occurs at moderately low humidities.

An air disinfectant recommended which is free from influence by the humidity factor is lactic acid sprayed in the proportion of 10 mgm. per cubic metre: it acts over a wide range of relative humidity, 40 to 85 per cent. (see Lovelock, J. E., Lidwell, O. M., and Raymond, W. F., *Nature*, 1944, Jan., p. 20).

For further information on the subject of air disinfection the following papers may be consulted: ultra-violet radiation —Wells, W. F., *et al., Amer. Journ. Hygiene*, 1942, **35**, 97; Del Mundo, F., and McKhann, C. F., *Amer. Journ. Diseases Child.*, 1941, **61**, 213; Robertson, E. C., *et al., Journ. Amer. Med. Assoc.*, 1943, **121**, 908; propylene glycol—Robertson, E. C., *et al., Science*, 1941, **93**, 213 and **94**, 612; *Journ. Exper. Med.*, 1942, **75**, 593; Harris, T. N., and Stokes, J., *Amer. Journ. Med. Sci.*, 1943, **206**, 631; hypochlorous acid and Influenza virus—Edward, D. G. Ff., and Lidwell, O. M., *Journ. Hygiene*, 1943, **43**, 196.

NEW REGULATIONS FOR THE CONTROL OF HEAT-TREATED AND PASTEURISED MILK

England and Wales.

Under Regulation 55 G of the Defence (General) Regulations, 1939, it is laid down that both "Tuberculin-tested (pasteurised)" and "Pasteurised" milk, in addition to the requirements under the Regulations of 1936 to 1943 (*vide* pp. 305 and 306), must conform to the following tests now prescribed for "Heat-treated milk": *i.e.* when tested under the prescribed conditions must not give a reading of more than 2·3 Lovibond blue units on examination by the phosphatase test (*vide infra*), and must not reduce methylene blue within thirty minutes after being kept in the laboratory at atmospheric shade temperature not exceeding 65° F. till between 9 and 10 a.m. in the day following that in which it is received (*vide* p. 308).

Scotland.

See: The Heat Treatment of Milk (prescribed tests) Order (Scotland), 1944; and The Milk (special designations) Amendment Order (Scotland), 1944; H.M. Stationery Office, London.

PHOSPHATASE TEST FOR HEAT-TREATED MILK [1]

This test determines the inactivation of the enzyme phosphatase, normally present in cow's milk, by such degree and time of heating as to destroy non-sporing pathogenic organisms, *e.g.* 62·8° C. (145° F.) for thirty minutes as in the standard method of pasteurisation. The presence of the enzyme is detected by its ability to liberate phenol from disodium phenyl-phosphate, the phenol being estimated colorimetrically with Folin and Ciocalteu's reagent which yields a blue coloration, and the result is expressed in arbitrary units; thus a deep blue colour, *i.e.* over 6 units, indicates a large amount of phenol; a pale blue, *i.e.* under 2·3 units, a small amount.

Reagents required.

(1) Buffer substrate tablets (disodium phenyl-phosphate and sodium barbitone [2]: dissolve one tablet in 50 c.c. boiling distilled water, boil for one minute and cool rapidly. This solution must be freshly prepared.

(2) Folin and Ciocalteu's phenol reagent.[2] This solution is diluted with twice its volume of distilled water before use. The reagent must be protected from contact with dust and any reducing substances, and should be stored in the refrigerator.

(3) Fourteen per cent. aqueous solution (weight/volume) of pure anhydrous sodium carbonate (Analar).

Two tests are available, a "short test" (test A) which is useful for detecting gross errors in pasteurisation, and a "long test" (test B) which is recommended for routine use.

Test B (long test).

(*a*) *Method.*

To 10 c.c. of the buffer substrate solution in a 25 c.c. stoppered test-tube marked at 10 c.c. (*e.g.* as used for methylene blue reduction tests), add 0·5 c.c. of the well-mixed milk and mix thoroughly. Add 3 drops of chloroform, stopper the tube, and incubate for 24 ± 2 hours at 37°–38° C. At the end of this time cool, add 4·5 c.c. of the diluted Folin-Ciocalteu reagent, mix, allow to stand for three minutes, and filter, using a Whatman filter paper No. 40 (or No. 30). To 10 c.c. of the filtrate add 2 c.c. of the sodium carbonate solution, mix, place the tube in a boiling water-bath (kept boiling) for five minutes and again filter. Compare the colour in a Lovibond comparator with the series of colour standards (on the appropriate "phosphatase" disk), each of which represents a given unitage: 6, 2·3, 1·5 and 0·5.

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[1] Kay, Aschaffenburg & Neave, "The phosphatase test for control of efficiency of pasteurisation." *Imperial Bureau of Dairy Science, Technical Communication No.* 1, October, 1939. See also Statutory Rules and Orders, 1944, No. 349, Emergency Powers (Defence), Milk and Dairies, England and Wales, The Heat-treated Milk (prescribed tests) Order, 1944, H.M. Stationery Office, London.

[2] Obtainable from British Drug Houses, Ltd.

(*b*) *Controls.*

The test on each milk sample should be made in duplicate, and the following control must be included along with each set of tests:—a "blank" in which the reagents only, without milk, are tested as above. Milk samples are also kept in the refrigerator for twenty-four hours after the tests have been put up, and if a sample has given a reading of over 2·3 units (*vide infra*) then the test is repeated but *omitting incubation.*

(*c*) *Interpretation.*

The "blank" should give a reading of not more than 0·5 Lovibond blue units, and the control test omitting incubation not more than 1·5 units. With such control results, milks which give readings of 2·3 Lovibond blue units or less are classified as "properly pasteurised" or "giving a negative phosphatase test"; those giving readings between 2·4 and 6·0 units are classified as "improperly pasteurised"; while those milks with a reading of more than 6·0 units should be reported as "grossly underpasteurised."

Where possible, the milk should be tested within eighteen hours of pasteurisation. The test is extremely sensitive, and great care is necessary in the cleaning of all glassware, which should be placed in glass-cleaning solution, and kept apart from phenol-containing substances, *e.g.* lysol and coal-tar soaps. The rubber stoppers must be tested for phenolic impurities before use. Care must be taken not to contaminate pipettes with saliva, and a separate pipette must be used for each sample.

By diluting the Folin and Ciocalteu's reagent with two volumes of 5 per cent. sodium hexametaphosphate instead of distilled water, and by keeping the tubes in the boiling water-bath for two instead of five minutes, the second filtration can be avoided.

TITRATION OF BACTERIOSTATIC ACTION OF BLOOD DURING PENICILLIN TREATMENT[1]

When systemic treatment with penicillin is carried out its bacteriostatic action in the blood should be estimated to ensure that an adequate content of penicillin is being maintained.

The following method, which has been used in the Bacteriology Department, Edinburgh University, is recommended.

Blood is withdrawn by vein puncture and the serum separated in the usual way, being centrifuged if necessary so that it is completely free from suspended red cells. In drawing blood and carrying out the necessary manipulations all the necessary precautions should be taken to avoid contamination.

The bacteriostatic test is carried out as soon as possible after withdrawal of the blood, but reliable results can be obtained with serum kept at 0°–4° C. for periods up to twenty-four hours.

To each of a series of sterile stoppered tubes ($3 \times \frac{1}{2}$ inch) 0·3 c.c. of varying concentrations of the serum is added as follows :—

	1	2	3	4	5
Serum :	undiluted.	dil. 1 in 2	1 in 4	1 in 8	1 in 16

The dilutions are made in sterile broth and are prepared with graduated pipettes as in serological tests involving the preparation of a series of doubling dilutions (*vide* p. 250 *et seq.*) ; pipettes used must be sterile, the usual technique being adopted to ensure sterility of all materials employed. A control tube containing 0·3 c.c. of broth is included in the series. To each tube is then added a large loopful (standard loop of 3 mm. diameter), of a 1 in 300 dilution (in broth) of a twenty-four hours' broth culture of a standard strain ("Oxford H") of *Staphylococcus aureus.*

The tubes are incubated for eighteen to twenty-four hours, when readings of the resulting growth, or absence of growth, are made according to the visible turbidity or absence of turbidity.

In this way the bacteriostatic level of the blood serum can be determined within twenty-four hours. If necessary these results can be confirmed or checked by taking a loopful from each tube after thorough shaking of the contents, and by making single stroke inoculations in parallel on a 4-inch plate of agar or blood-agar, the position of each stroke being indicated by dividing the outside of the plate into divisions with a grease pencil. The plate will accommodate six such divisions, one for the stroke inoculation from each tube. The presence or absence of growth in the tubes and the relative amount of growth when there is partial inhibition is thus conclusively determined after twenty-four hours' incubation of the plate.

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[1] For micro-methods of estimating penicillin in serum and other body fluids, the reader should consult the following recent papers: Fleming, A., *Lancet*, 1944, ii, 620; and Fleming, A., Young, M. Y., Suchet, J., and Rowe, A. J. E., *Lancet*, 1944, ii, 621.

It should be noted that normal serum may produce some degree of bacteriostatic action, though this is usually slight, and it is therefore necessary to test the serum (as above) before the commencement of penicillin treatment. This result serves as a basis for comparison.

It may be said that in general satisfactory systemic administration of penicillin will yield results in the bacteriostatic test of the order of those indicated below, the relative amount of growth being indicated by the number of + marks.

	1	2	3	4	5
Serum :	undiluted.	dil. 1 in 2	1 in 4	1 in 8	1 in 16
	—	—	— to ++	+ to +++	++ to +++

With normal serum the *usual* results are as follows :—

1	2	3	4	5
+ or ++	+++	+++	+++	+++

An approximate estimate of the penicillin content of undiluted patient's serum in the bacteriostatic test may be obtained from consideration of the following data : one unit of penicillin in 20 c.c. of nutrient broth usually inhibits completely the growth of the standard strain of *Staph. aureus* used in these bacteriostatic tests and in the assay tests on penicillin solutions as carried out in the Bacteriology Department, University of Edinburgh. If it is *assumed* that one unit of penicillin in 20 c.c. of patient's serum has approximately the same inhibitory power against the standard strain of *Staph. aureus* as one unit of penicillin in 20 c.c. of broth, it may be stated that when undiluted serum completely inhibits the growth of the test organisms in the bacteriostatic tests, then there is approximately $\frac{1}{20}$th of a unit of penicillin present in 1 c.c. of the serum ; when the inhibition titre is 1 in 2 there is approximately $\frac{1}{10}$th of a unit in 1 c.c. of serum ; when the titre is 1 in 4, $\frac{1}{5}$th unit, and so on. Estimations of the penicillin content of serum based on the considerations outlined above must be regarded as approximate only since factors such as the inhibiting power of the serum itself against the test organisms may obviously influence the result.

TESTING OF SENSITIVITY OF BACTERIA TO SULPHONAMIDE COMPOUNDS AND TO PENICILLIN

Before commencing treatment with penicillin the sensitivity of the infecting organisms present in the case should be ascertained. This can be accomplished by a technique (originally used by Fleming) which can also be employed for testing sensitivity or resistance of organisms to the sulphonamide compounds. Thus in sulphonamide therapy it may be found that the clinical response is unsatisfactory and this may be due to the fact that the particular infecting strain, *e.g.* of *Strept. pyogenes*, is sulphonamide-resistant.

Sulphonamides.—A strip of agar about ½ inch wide is cut from the centre of a plate of suitable medium (usually blood agar) and discarded. Agar (5 c.c.) containing an appropriate amount of the sulphonamide (20 mgms. per cent., *i.e.* 1 in 5000) is melted in a tube and cooled to about 50° C., mixed thoroughly with 0·25 c.c. of horse blood and pipetted into the gutter in the plate of medium. The plate is then placed in the refrigerator overnight in order to allow diffusion of the sulphonamide into the medium, after which it is inoculated with a suitable dilution of the organism, e.g. *Strept. pyogenes*, to be tested. A satisfactory inoculum is a small loopful of a twenty-four hour culture in serum broth diluted 1 in 2000. The inoculation is made by stroking across the plate at right angles to the gutter; it is desirable to control the test by similarly inoculating with known sensitive and/or resistant strains. With sensitive organisms growth does not occur right up to the edges of the gutter but is inhibited for a distance varying with their sensitivity to the sulphonamide under examination; resistant organisms are not inhibited (or only to a much smaller extent).

Penicillin.—The gutter may conveniently be cut near the edge of the plate (agar or blood agar) which is inoculated with the test organisms and also with a strain of *Staphylococcus aureus* of known sensitivity to penicillin. It is unnecessary to leave the plate overnight in the refrigerator after penicillin is added to the gutter (*cf.* sulphonamides). Inoculation is carried out at right angles to the gutter across the plate, starting at the edge of the gutter which is then almost filled with a solution of penicillin containing about 1–2 units per c.c. When a fluid preparation (as above) is used in the gutter it is an advantage, before introducing the solution, to seal the lower edges of the gutter to the Petri dish by contact with a hot metal rod. After incubation for eighteen to twenty-four hours at 37° C. with the lid of the plate uppermost, the plate is examined for inhibition of growth as in the tests with sulphonamides. Pencillin-sensitive strains of *Staphylococcus aureus* are inhibited for a distance of approximately ½ inch under the conditions outlined above.

The penicillin may be incorporated with melted agar cooled to 45° C. and the mixture used to fill the gutter, but this is not essential. If desired, the control organisms may be of the same species as that isolated from the patient (*e.g.* a streptococcus, etc.), but reliable comparative results may be obtained by using as a control a *Staphylococcus aureus* of known sensitivity to penicillin.

The size of the inoculum is relatively unimportant in testing penicillin-sensitivity and approximately similar results have been obtained by using a loopful of an undiluted fluid culture, or of a 1 in 400 dilution (in broth) of the culture, or by taking the inoculum from a single colony of a plate culture.

When testing for sensitivity either to penicillin or to sulphonamides the medium used should not be less than about 3 mm. in thickness.

More detailed and accurate observations upon sensitivity to penicillin (*e.g.* possible changes in sensitivity of the infecting organisms during penicillin treatment) may be carried out by repeatedly observing the range of inhibition of the organism with graded dilutions of penicillin.

CHAPTER X

SEROLOGICAL TYPES OF THE PNEUMOCOCCUS

SINCE the recognition of the numerous types of the original Group IV, the occurrence of cross-reactions has been observed, and it has been found that precise determination of types may only be made by means of sera absorbed with cross-reacting strains. Recent serological analyses have thus resulted in sub-types and new types being defined and have extended the total number of types (including sub-types) to seventy-five. A system of grouping related pneumococcus types for the production of polyvalent diagnostic and therapeutic sera has also been suggested (see Eddy, *Pub. Health Rep.*, Washington, 1944, **59**, 449, 451, 1041).

CHAPTER XII

THE RECOGNITION OF COLONIES OF THE DIPHTHERIA BACILLUS ON HOYLE'S MEDIUM[1]

(See page 387)

After twelve hours' incubation.—To the naked eye growths of the *gravis* and *mitis* types represent a grey " haze " hardly distinguishable from growths of diphtheroid bacilli though the latter are somewhat blacker and more glistening. Magnified and by reflected light the colonies are matt and not smooth like those of diphtheroid organisms. The *Intermedius* type show very tiny colonies which are strikingly uniform in size and appearance.

After twenty-four to forty-eight hours.—*Gravis type*—seen by daylight growths are slate-grey with a bluish tinge ; individual colonies have a paler border and if well separated attain a diameter of 3 mm. after thirty-six to forty-eight hours. Magnified and by reflected light from an electric bulb the surface of the colonies has a ground-glass appearance which only indistinctly reflects the image of the bulb ; touched with a wire and observed with the plate culture microscope the colonies are seen to break up very readily ; the shape may approximate to the daisy-head formation seen on McLeod's medium (p. 375), but often the colonies show merely a radial striation sloping from the raised centre to the slightly crenated periphery ; sometimes the colonies are domed and circular in outline with only slight striation and crenation.

Mitis type—the colour, consistence and size of colonies is similar to those of the *gravis* type and they also show a ground-glass appearance though more glistening ; the colonies, however, are domed and have a perfectly circular outline.

Intermedius type—the colonies are never larger than 2 mm. and growths are more delicate than those of the other types ; magnified and by transmitted light, the colonies are very uniform in size and seem " pricked out " on the surface of the medium ; they are blacker than the other types, but are of the same roughness and consistency ; they are usually domed and circular with a tendency to crenation, but occasionally the margin is flattened and they show a " poached egg " appearance ; after forty-eight hours they are frequently papillate.

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[1] See Wright, H. A., *Edin. Med. Journ.*, 1944, **50**, 737.

It should be noted that these colonies are sometimes difficult to distinguish from those of certain streptococci growing from throat swabs, but the latter are black or brown and somewhat flatter. (A microscopic examination should be made if there is any doubt.)

Diphtheroid bacilli.—Growths are generally more glistening than those of the diphtheria bacillus ; magnified and by reflected light colonies usually have a smooth surface on which the image of the electric bulb is sharply delineated ; in colour they range from black, dark-brown to pale grey or greyish-white ; the characteristic slate-grey colour of the *gravis* type of diphtheria bacillus is seldom seen ; colonies are sometimes tough or mucoid in consistence, or soft and butyrous.

It may be noted that a Gram-positive micrococcus occasionally growing from throat swabs may resemble the *gravis* type, but is more rugose and glistening. Another type of micrococcus may form a daisy-head colony, but is more glistening than the *gravis* type of colony. (Microscopic examination should be made if there is any doubt about such colonies.)

In examining plates a uniform procedure should be used throughout since the precise appearance and colour vary with the nature of the illuminant and the angle of the light. Colour is best seen with the naked eye in the confluent parts of the growth. It must also be remembered that variations in batches of medium as regards, for example, moisture, sample of tellurite, type of peptone, and the age of the medium, may influence colony characters ; and in diagnostic work it is advantageous to use for comparison plates on which known strains of *gravis*, *mitis* and *intermedius* types have been inoculated and grown at the same time as the cultures under examination.

CHAPTER XVI

SEROLOGICAL TYPES OF VIBRIO CHOLERAE

It may be noted that within the serological subgroup represented by the classical *V. cholerae* and with a common O antigen, serological types can be recognised, distinguished by agglutination and absorption tests with O antisera ; this difference depends on a subsidiary O antigenic component characteristic of the type ; the stability of these types is doubtful.

In India two such types have been recognised and designated according to the names of standard strains, "Inaba" and "Ogawa."

It is possible that a third such type also exists possessing the characteristic components of both the above-mentioned types (see Gardner, A. D., and Ventratraman, K. V., *Lancet*, 1935, i, 265).

It is also of interest that similar type differences occur among haemolytic (El Tor) vibrios.

CHAPTER XVII

BACTERIOLOGICAL DIAGNOSIS OF WHOOPING-COUGH

On p. 500 reference is made to the isolation of *B. pertussis* by the "cough-plate" method which has proved of practical value in the recognition of early cases of whooping-cough before the typical clinical manifestations have declared themselves. This method, while applicable to hospital cases, is less convenient in general medical practice ; it is also subject to the disadvantage that frequently there is such an excessive growth of other organisms from the respiratory passages that *B. pertussis* colonies are overgrown or difficult to recognise.

More recently it has been recommended that a specimen for bacteriological diagnosis can be obtained more conveniently by using a post-nasal swab. For this purpose the pledget of cotton-wool is mounted on a long flexible wire and the swab is passed into the post-nasal space from the anterior nares. In preference to this method, Cruickshank (see *Lancet*, 1944, i, 176) has successfully used the ordinary throat swab bent at the end (pp. 321, 366) and introduced behind the soft palate so as to collect secretion from the naso-pharynx. In cultivating the organism a plate of the Bordet-Gengou medium, as described on p. 149, is inoculated by smearing a section of the plate directly with the swab and then distributing the inoculum from this area on the remainder of the surface with an inoculating wire (p. 366). To prevent overgrowth of the plate by other organisms and to render colonies of *B. pertussis* more easily detectable and facilitate isolation of pure growths, the surface of the medium is previously impregnated with about four drops of a suitable dilution of *penicillin* (pp. 498 and 500). This dilution is ascertained for each penicillin preparation by testing its inhibitory action towards various Gram-positive and Gram-negative organisms. (According to Cruickshank, 15–25 Oxford Units—*vide* p. 656—per 12 c.c. of medium (4-inch plate) is a suitable concentration for the isolation of *B. pertussis.*) The penicillin should be added half an hour before inoculating the plate and spread evenly with a sterile glass spreader (p. 168), and the surface of the medium is then dried in the incubator. It is claimed that more numerous colonies are obtained from the naso-pharyngeal swab than by the cough-plate method and it has also been shown that if swabs are kept moist by means of a small amount of saline-agar at the foot of their glass containers, the organisms remain viable for twenty-four hours (p. 322).

CHAPTER XVIII

THE BACTERIOLOGICAL EXAMINATION OF INFECTED WOUNDS, WITH SPECIAL REFERENCE TO INFECTION BY SPORING ANAEROBES

A GREAT variety of bacteria may flourish in a wound: *Streptococcus pyogenes*, pathogenic staphylococci (coagulase-positive), e.g. *Staphylococcus aureus*, the various sporing anaerobic bacilli (the tetanus and gas-gangrene organisms), streptococci of the *viridans* type, enterococcus, anaerobic or microaerophile streptococci, pneumococcus, *B. proteus*, *B. pyocyaneus*, coliform bacilli, *B. diphtheriae*, haemophilic bacteria, non-pathogenic staphylococci (coagulase-negative) and various saprophytic Gram-positive cocci, various Gram-negative cocci, *B. faecalis alkaligenes*, non-pathogenic diphtheroid bacilli, sporing aerobic bacilli (*B. subtilis* group). Though some of these are mainly saprophytic and are of little clinical significance, their presence may delay healing.

When a systematic bacteriological investigation of a wound is called for, and particularly with a view to ascertaining the effects of treatment, repeated examinations are required starting from the time of admission to hospital or just before any initial operative procedure, the second examination being made when the first dressing is carried out and subsequent examinations at weekly intervals or whenever there is any adverse change in the clinical condition. (See Medical Research Council, War Memorandum No. 2, Revised Second Edition. London: H.M. Stationery Office, 1943.)

Specimens of exudate should be taken from the wound, particularly from the deeper parts and from parts where the infection seems to be most pronounced. These may be obtained in capillary tubes, but sterile swabs (similar to throat swabs, p. 321), rubbed over the wound surface and soaked in the exudate, serve well for the purpose. Sterile swabs mounted on wooden applicators, 5 inches long, are recommended in the memorandum cited above ; they are supplied to the surgical ward or theatre in a sterile container. Two or three swabs should be taken from the wound, one of which is used for film preparations, the others for culture. If there are sloughs or necrotic tissue present in the wound, small pieces should be placed in a sterile screw-capped bottle and used for microscopic examination and culture. When specimens are taken from wounds treated locally with a sulphonamide powder, the exudate before cultivation should be suspended in broth containing 0·05 per cent. *p*-aminobenzoic acid ; this neutralises any inhibition of growth due to the presence of the sulphonamide in the specimen.

Microscopical examination.—Films are made in the usual way and stained by Gram's method. These give some general picture of the degree and nature of the infection, *e.g.* the morphological types of organisms present (staphylococci, streptococci, Gram - negative or Gram - positive bacilli) and are of value in determining in a preliminary way if there is infection by the gas-gangrene anaerobes. Thus, if gas-gangrene is present, Gram-positive bacilli predominate and are also fairly numerous though it has been pointed out that *B. oedematiens* may appear to be relatively scanty in the wound exudate, even in an active infection. Thick rectangular Gram-positive bacilli would suggest the presence of *B. welchii*, *B. fallax*, or *B. bifermentans* ; boat or leaf-shaped pleomorphic bacilli with irregular staining may indicate *Vibrion septique* ; slender bacilli with round terminal spores suggest *B. tetani* or *B. tertius* ; *B. oedematiens* occurs in the form of large bacilli with oval subterminal spores.

Cultures.—The following media should be inoculated : (*a*) blood-agar plate to be incubated aerobically ; should *B. proteus* be present it tends to spread all over the plate, making impossible the isolation and recognition of other organisms : this may be obviated by incorporating potassium tellurite in the medium (p. 352) or by using 5 or 6 per cent. agar. (*b*) Blood-agar plate to be incubated anaerobically : the surface should be well dried before inoculation to prevent spreading of colonies of certain anaerobes. (*c*) Plate of MacConkey's medium. (*d*) Two tubes or bottles of cooked-meat medium ; after inoculation one is heated for thirty minutes at 65° C. to kill non-sporing organisms. (For the cultivation of *B. oedematiens* it has been recommended that a third tube or bottle should be inoculated with a broth emulsion of exudate or tissue which has been heated for five to ten minutes at 100° C..) (*c*) Litmus milk, in a long narrow tube, previously boiled and then cooled; after inoculation a layer of melted vaseline is superimposed on the milk.

After eighteen to twenty-four hours' incubation the aerobic blood-agar culture is examined and the various types of colonies scrutinized with the naked eye, a hand lens or plate culture microscope, and films may also be made and stained by Gram's method. If necessary the incubation may be continued to allow colonies to become more characteristic. In this way, for example, staphylococci and streptococci can be recognised and also the cultural types of these. Pure cultures can be obtained by subculture from isolated colonies, and staphylococci can be tested for the coagulase reaction, while streptococci can be examined if necessary for haemolysin production and serological characters. Various other aerobic organisms can also be identified from the blood-agar plate by appropriate methods.

The plate of MacConkey's medium is useful for the recognition at sight of lactose-fermenting coliform bacilli and the enterococcus, and also for ascertaining the relative numbers of coliform bacilli and *B. proteus* which forms pale colonies on this medium without spreading. Colonies of *Staphylococcus aureus* are easily identified on MacConkey's medium[1] by their characteristic colour—the usual yellow pigment being tinted pink by the acid change of the neutral red.

The anaerobic plate is examined after twenty-four and forty-eight hours' incubation. It must be remembered that this plate yields growths of various aerobes as well as the anaerobes. Comparison of the aerobic and anaerobic plates affords some indication of the presence of strictly anaerobic organisms in the wound exudate, but any suspected anaerobe must later be tested in subculture to ensure that it is unable to grow under aerobic conditions. (It may be noted that *B. tertius* and *B. histolyticus* can grow to some extent under aerobic conditions.) The colony characters of suspected anaerobes on the blood-agar plate are carefully studied with the naked-eye and plate culture microscope, and films are made and stained by Gram's method; this may give some preliminary information as to the type of anaerobe. Each type present must be isolated in pure culture for further examination, *e.g.* fermentation tests and animal inoculation. The difficulties of obtaining pure cultures of these organisms have been dealt with on p. 510, and the identification of the more important species is detailed under the heading of each organism described in Chapter XVIII. A table of differential characters is given on pp. 688 and 689.

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[1] For this purpose the medium should incorporate a good-quality peptone.

The litmus milk culture is intended for the rapid detection of *B. welchii* by the "stormy clot" reaction; but all strains do not give the reaction (*vide infra*—Nagler's reaction).

In the cooked meat medium both aerobes and anaerobes flourish, but the culture is useful for later subculture should the plate cultures fail to yield successful isolation of organisms present in the wound. This medium is specially valuable for anaerobes which grow slowly in culture and for the detection of small numbers of streptococci. Film preparations also yield further information as to the morphological types of organisms growing in it. For isolating the organisms present subcultures are made on aerobic and anaerobic blood-agar plates and these are studied in the usual way.

Additional Methods.

If anaerobic jars are not available, anaerobes may be isolated in deep agar shake cultures. Four or five serial decimal dilutions of the exudate are prepared in broth and each of these is used to inoculate melted agar kept at 45° C., which is then allowed to solidify in tubes. After incubation, one of the dilutions will show colonies sufficiently separate to allow of single-colony subculture by means of a Pasteur pipette after cutting the tube transversely. A convenient alternative method is to take up the melted agar after inoculation in sterile Pasteur pipettes stoppered with cotton-wool, the capillary end being then sealed and the pipettes incubated horizontally.

Certain reducing agents have recently been used for rendering fluid media anaerobic in the bacteriological examination of wounds, etc.—ascorbic acid (0·1 per cent.), thioglycollic acid (0·02 per cent.), reduced iron and iron strips (*vide* p. 664), and these may be adopted advantageously in routine work with the anaerobic organisms.

Nagler's reaction.—This depends on the demonstration of the lecithinase activity of the α toxin of *B. welchii* with the splitting of a soluble lipo-protein in human serum and the formation of a precipitate of lipoids and protein. The effect is specifically neutralised by *B. welchii* antitoxin. In the test equal parts of Fildes' peptic digest broth (p. 508) and sterile human serum (from clotted blood) are mixed, and 0·3 c.c. of this mixture is placed in each of two small stoppered tubes, to one of which 0·03 c.c. of a standard *B. welchii* antitoxin has been added. Both tubes are then inoculated with a drop of a

SOME DIFFERENTIAL CHARACTERS OF ANAEROBIC BACILLI *(Clostridia)*

	Morphology in culture *	*Colonies on blood-agar*	*Cooked-meat medium*	*Milk medium*	*Liquefaction of coagulated serum*	*Fermentation of*				*Pathogenicity to guinea-pigs and mice*
						Glucose	Lactose	Saccharose	Salicin	
B. welchii	Large, thick, often rectangular bacilli; spores usually absent	Large, circular, with regular outline; haemolytic	Gas, no digestion, meat reddened	Acid, gas, rapid clotting; "stormy-clot"	—	+	+	+	$\left(\begin{smallmatrix}-\\+\end{smallmatrix}\right)$	+
Vibrion septique	Large bacilli with central or subterminal spores †	Transparent, irregular, with spreading projections; usually haemolytic	Gas, no digestion	Acid, gas, slow clotting	—	+	+	—	+	+
B. oedematiens	Like *B. welchii* but somewhat larger and more pleomorphic; central or subterminal spores (not numerous)	Transparent, flat, tend to fuse and form spreading film; usually haemolytic	Gas, no digestion	Acid, gas, sometimes slow clotting	—	+	—	—	—	+
B. tetani	Slender bacilli with round terminal spores	Transparent with long feathery spreading projections; usually haemolytic	Slight digestion, blackening and putrefactive odour	Unaltered	— (but may be softened)	—	—	—	—	+ (tetanus produced)

B. tertius	Long, slender bacilli, with oval terminal spores	Small, transparent, with regular outline; non-haemolytic	Gas, no digestion	Acid, gas, slow clotting	—	+	+	+	+	—
B. fallax	Resembles *B. welchii*	Large, opaque irregular	Gas, no digestion	Acid, gas, slow clotting	—	+	+	+	+	+ when first isolated
B. sporogenes	Somewhat slender bacilli; central or subterminal spores	"Medusa-head" formation or irregular, with feathery projections; haemolytic	Gas, digestion, blackening and putrefactive odour	Acid, clot, digestion, later alkaline	+	+	—	—	$\left(\frac{-}{+}\right)$	—
B. histolyticus	Resembles *B. sporogenes*	Small, transparent, circular or irregular; non-haemolytic	Digestion, blackening and putrefactive odour; deposit of tyrosine crystals	Clot and digestion	+	$\left(\frac{\perp}{-}\right)$	—	—	—	Usually +
B. tetanomorphus	Resembles *B. tetani*; round terminal spores	Small, circular	Gas; no digestion; no putrefactive odour	Unaltered	— (but may be softened)	+	+	—	—	—

See also table of characters of anaerobic bacilli in Medical Research Council War Memorandum No. 2 Revised Second Edit., 1943.

* All these organisms are Gram-positive, but Gram-negative forms are seen in older cultures; they are all motile with peritrichous flagella except *B. welchii* and *B. fallax*, but motility is not pronounced and has to be observed while the organisms remain in an anaerobic environment, *e.g.* withdrawn from a young anaerobic culture into sealed capillary tubes.

† Morphological forms seen in tissues are referred to in the text (*vide supra*).

+ Under fermentation signifies acid and gas production.

⊥ Under fermentation signifies acid production without gas.

fluid culture or a colony picked from a plate culture. The tubes are incubated anaerobically at 37° C. and examined after sixteen, forty and sixty-four hours. A positive reaction is indicated by the development of pronounced turbidity in the serum with a yellowish curd on the surface, the effect being absent in the tube containing antitoxin.

This reaction has also been utilised for the rapid detection of *B. welchii* in direct plate culture, and allows a serologically controlled identification of the organisms to be made within twenty hours of inoculating the plate from the wound exudate. The method is described in the Medical Research Council Memorandum cited, p. 684. 2·65 c.c. of human serum are mixed with 0·65 c.c. of Fildes' peptic digest broth; the temperature is raised to 50° C. and 10 c.c. of melted nutrient agar at 50° C. are added. A plate is poured, allowed to set and dry. On one-half of the plate (which is appropriately marked) two or three drops of standard *B. welchii* antitoxin are spread and allowed to dry. The whole plate is then inoculated from the wound swab. On the section containing no antitoxin, *B. welchii* colonies show a surrounding zone of opacity, *i.e.* the Nagler reaction, while colonies of the organism on the remainder of the plate show no change.

CHAPTER XXIII

FIELD'S RAPID METHOD OF STAINING THICK BLOOD FILMS FOR MALARIA PARASITES[1]

This method can be recommended for routine use.

In preparing the blood films it is important to ensure that they are not too thick. Drying may be assisted by placing the film in the incubator. After the film is quite dry it may be passed very rapidly two or three times through a Bunsen or spirit flame, each passage occupying two to three seconds. When cool the film is ready for staining.

Field's Stain[2]

Solution A. (Methylene blue.)

Methylene blue	1·3 gms.
Disodium hydrogen phosphate (anhydrous)	5·0 gms.
(If crystalline salt is used, 12·6 gms.)	

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[1] *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1941, **35**, 35.

[2] This stain in tablet form can be obtained from G. T. Gurr, London.

Dissolve in 50 c.c. distilled water, bring to the boil and evaporate almost to dryness in a water bath, then add potassium dihydrogen phosphate (anhydrous) 6·25 gms. (crystalline salt 8·0 gms.) Add 500 c.c. of freshly boiled and still warm distilled water, stir until the stain is completely dissolved and set aside for twenty-four hours. Filter before use. If a scum forms during use, filter again.

Solution B. (Eosin.)

Eosin	1·3 gms.
Disodium hydrogen phosphate (anhydrous) (Crystalline salt, 12·6 gms.)	5·0 gms.
Potassium dihydrogen phosphate (anhydrous) (Crystalline salt, 8·0 gms.)	6·25 gms.
Distilled water	500 c.c.

The phosphate salts are first dissolved in freshly boiled and still warm distilled water, then the stain is added. Set aside for twenty-four hours and filter before use.

The stains are kept in covered jars, the level being maintained by the addition of fresh stain as necessary. The same solution may be used continuously for many weeks without apparent deterioration, but the eosin solution should be renewed when it becomes greenish from the slight carry over of methylene blue. If solutions show a growth of bacteria or moulds they should be discarded and replaced from stock solutions which if stored carefully will remain satisfactory up to a year.

Method of Staining.

(1) Dip the slide into the Solution A for one to two seconds only.

(2) Remove slide and immediately rinse *gently* in a jar of clean distilled or tap water until the stain ceases to flow from the film and the glass of the slide is free from stain.

(3) Dip the slide into Solution B for one to two seconds only.

(4) Rinse *gently* for two to three seconds in clean water.

(5) Place *vertically* against a rack to drain and dry.

The relative times may require slight adjustment to suit different batches of stain.

Films up to three weeks old may benefit from immersion in phosphate buffer solution (as used for dissolving the stains) until haemoglobin begins to diffuse out. The film is stained in the ordinary way. Unduly thick films should be similarly immersed before staining to remove the greater part of the haemoglobin. The phosphate buffer solution may be used in place of water for rinsing between *Solutions A* and *B*.

CHAPTER XXIV

THE PAUL-BUNNELL REACTION IN THE DIAGNOSIS OF INFECTIVE MONONUCLEOSIS

This diagnostic test has been referred to briefly on p. 635. It depends on the agglutination of sheep red cells by a " heterophile " antibody in the patient's serum, *i.e.* an antibody specific for an antigen having no apparent biological relationship to that acting as the immunising stimulus in the disease. A similar reaction may, however, occur with normal serum in low titre, and it is essential in carrying out the diagnostic test to differentiate between such normal serum reaction and the effect which is characteristic of infective mononucleosis. Moreover, in persons who have recently received an injection of a therapeutic serum (from the horse), *e.g.* cases of serum sickness, an apparently similar heterophile antibody may be present in considerable amount in the blood, since horse serum contains the appropriate heterophile antigen and stimulates the production of an antibody for sheep red cells. It may be noted here that the original heterophile immunity phenomenon described by Forsmann was the production in the rabbit of an antibody for sheep red cells by the injection of guinea-pig kidney tissue, and it is now known that the serum and tissues of other animal species, *e.g.* horse, act in the same way, each possessing a common heterophile antigen.

It has been shown that the type of antibody present in infective mononucleosis differs in certain respects from the Forsmann antibody, and also from that found in normal serum, and that this difference can be determined by agglutinin-absorption tests as follows :—

Antibody present in—	*Treated with emulsion of guinea-pig kidney.*	*Treated with ox red cells.*
Normal serum.	Absorbed.	Not absorbed.
Serum sickness.	Absorbed.	Absorbed.
Infective mononucleosis.	Not absorbed.	Absorbed.

Method of Testing for Absorption (Barrett)[1]

Guinea-pig kidney emulsion.—Carefully washed and finely sieved kidney tissue is autoclaved and suspended in 0·5 per cent. phenol-saline, the proportion of tissue in the suspension being 1 in 6 (by volume).

Ox red cell suspension.—Washed ox cells are suspended in three times their volume of saline, autoclaved and strained through muslin; sufficient saline is added to make up the proportion of cells in the suspension to 1 in 5; an equal volume of 1 per cent. phenol-saline is added. The suspension is further diluted 1 in 10 before use.

Test.—To 1 c.c. of serum add 0·3 c.c. of kidney suspension divided into three successive doses of 0·1 c.c., allowing each to act for forty minutes; centrifuge, pipette off supernatant fluid which is now a 1 in 1¼ dilution of the treated serum. Reserve 0·3 c.c. of this for the ox cell absorption test (*vide infra*) and dilute the remainder with an equal volume of saline making a 1 in 2½ dilution; reserve 0·5 c.c. of this and as before dilute remainder with an equal volume of saline, making a 1 in 5 dilution.

Carry out a preliminary agglutination test, adding 0·5 c.c. of 0·2 per cent. sheep red cells to 0·5 c.c. of the treated serum diluted 1 in 5. The tube containing the mixture is centrifuged (without previous incubation) at 2,000 r.p.m. for ten minutes, and if the cells can be re-suspended uniformly by gentle tapping of the tubes the result may be regarded as negative, i.e. *the antibody has been absorbed.* Such result, as shown above, is contrary to that usually observed in cases of infectious mononucleosis, and the Paul-Bunnell reaction may be regarded as negative.

If, on the other hand, the agglutination result is positive as indicated by macroscopic clumping of the cells, *the antibody being unabsorbed*, further testing is required to ascertain (1) the titre of the treated serum, and (2) whether the antibody can be absorbed by ox red cells. These tests are combined as follows:—Set out in a rack two rows (A and B) of 3 × ½ inch tubes so that tube 1 of A is opposite tube 4 of B (as shown below). Add 1 c.c. saline to each tube of A. In tube 1 of B place 0·3 c.c. of serum treated with kidney tissue, dilution 1 in 1¼, and in tubes 2 and 3 0·5 c.c. of dilutions 1 in 2½ and 1 in 5 respectively; 1 c.c. of the 1 in 5 dilution is also added to tube 1 of A, *i.e.* the tube now contains 2 c.c. of a 1 in 10 dilution. Transfer 0·5 c.c. of this to tube 4 of B and 1 c.c. to tube 2 of A which now contains 2 c.c. of a 1 in 20 dilution ; repeat the procedure by transferring 0·5 c.c. from 2A to 5B and 1 c.c. to 3A, and so on, thus making several doubling dilutions in both rows as follows :—

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[1] See Barrett, A. M., *J. Hygiene*, 1941, **41**, 330.

	1 in 1¼	1 in 2½	1 in 5	1 in 10	1 in 20	1 in 40	1 in 80	1 in 160	1 in 320	1 in 640
A	–	–	–	1	2	3	4	5	6	7
B	1	2	3	4	5	6	7	8	9	10

Final dilution after addition of blood :

1 in 2½ 1 in 5 1 in 10 1 in 20 1 in 40 1 in 80 1 in 160 1 in 320 1 in 640 1 in 1280

By adding to each tube in row A 0·5 c.c. of 0·2 per cent. sheep red cells, and proceeding as in the preliminary test, the titre of the serum treated with guinea-pig kidney can be ascertained.

To test for ox cell absorption the tubes in B are used. If the titre (in terms of the final dilution) in A is, for example, 1 in 40 (tube 2) then to tubes 1, 2 and 3 of B is added an equal volume of diluted ox cell suspension. These tubes are then centrifuged and 0·5 c.c. of the supernatant fluid from each is tested with 0·5 c.c. of 0·2 per cent. sheep red cells for agglutination as in the preliminary test. If no agglutination occurs in two of the three tubes it may be assumed that the antibody has been absorbed since the concentration of serum in tube 3 of B is higher than the concentration in tube 2 of A. Similarly, if the titre were 1 in 80 (tube 3) then tubes 2, 3 and 4 of B would be used. Similarly also for higher titres. A serum with a titre of at least 1 in 40 after treatment with guinea-pig kidney, which shows absorption with ox cells, is indicative of the reaction in infective mononucleosis.

In the absence of absorption tests the significance of low-titre reactions has to be assessed in the light of clinical data, any history of serum therapy, the stage of the disease and the haematological picture. Titres up to 1 in 128 may be given occasionally by normal serum (p. 635). However, repeated examinations may demonstrate a titre rising above 1 in 128 and so yield a more conclusive result.

If a second reading of results is made after the tubes have stood overnight at room temperature or in the refrigerator, they should be replaced at 37° C. for one to two hours. This avoids fallacious results from " cold agglutination " (p. 636) which is reversible at 37° C. and so far as is known is not associated with infective mononucleosis.

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